PRENATAL GLUCOCORTICOID PROGRAMMING OF 11-BETA HYDROXYSTEROID DEHYDROGENASE TYPE 2 AND ERYTHROPOIETIN IN THE KIDNEY

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DECLARATION OF ORIGINALITY

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Signature

Name

Dedicated to my wife

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ABSTRACT

Numerous epidemiological studies show a strong association between low birth weight and later life hypertension and metabolic disease. Excessive in utero exposure to glucocorticoids ('stress hormones') has been hypothesized to be important in such developmental 'programming', acting via crucial physiological, gene expression or structural changes in the developing fetus. Normally, the fetus is protected from the high levels of maternal glucocorticoids by an enzymic placental barrier, 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2). In the placenta, 11β-HSD2 efficiently converts active maternal glucocorticoids (cortisol in humans; corticosterone in rodents) to physiologically inactive 11-keto forms. In previous studies in rats, maternal administration of dexamethasone, a synthetic glucocorticoid which is minimally metabolized by 11β-HSD2, or carbenoxolone, a potent inhibitor of 11 β-hydroxysteroid dehydrogenase, increased glucocorticoid load to the fetus. This resulted in lower offspring birthweight and later life hypertension and hyperglycemia — important components of the metabolic syndrome. These programming effects were seen when dexamethasone was administered selectively during the third week of gestation.

We have used this well-validated model of programming to dissect the molecular mechanisms that mediate the programming of hypertension.

In accord with previous observations, administration of dexamethasone (100μg/kg/day) to pregnant rats during the last week of pregnancy significantly

reduced offspring birthweight by 10%. Moreover, the 9 month-old adult offspring had systolic hypertension (9% rise) accompanied by significant hypokalemia (10% fall K^+). The coexistence of hypertension and hypokalemia suggested that prenatal overexposure to dexamethasone might increase mineralocorticoid activity in the kidney. Intriguingly, although offspring of dexamethasone-treated dams had 46% lower plasma renin concentrations (consistent with intravascular fluid volume expansion), 24-hour total urinary aldosterone levels were significantly reduced compared to controls (reduction of 56%).

Maternal dexamethasone treatment was associated with a permanent decrease in 11β-HSD2 mRNA and activity in the kidney of the offspring (45% and 36% respectively). 11β-HSD2 plays an important role in regulation of renal sodium reabsorption (and thereby blood pressure) by acting as a pre-receptor barrier to MR access, preventing glucocorticoids from activating MR in the distal nephron. Thus, the decrease in renal 11β-HSD2 activity would allow greater endogenous glucocorticoids to activate MR, likely accounting for the low-renin, low-aldosterone hypokalemic hypertensive phenotype observed in these offspring.

Other components of mineralocorticoid or glucocorticoid signaling pathways, including mineralocorticoid receptor (MR), glucocorticoid receptor (GR) and 11-beta hydroxysteroid dehydrogenase type 1 (11β-HSD1) were not altered in the offspring kidney by prenatal glucocorticoid exposure. Dexamethasone-programmed offspring also showed exaggerated mineralocorticoid activity with increased kalliuresis in response to exogenously administered corticosterone, suggesting that the decrease in

renal 11β-HSD2 is functionally important. In this respect, our rat model resembles the syndrome of apparent mineralocorticoid excess where reduced 11β-HSD2 allows illicit activation of MR by glucocorticoids, resulting in excessive sodium reabsorption, hypertension and hypokalemia.

We also studied the effects of maternal dexamethasone on offspring erythropoietin expression in the kidney. This followed from previous observations that identified the hepatocyte nuclear factor 4 alpha (HNF4α) as a key gene up-regulated in dexamethasone-programmed offspring liver, where it might be involved in mediating hyperglycemia. HNF4α is also expressed in the kidney. The role of HNF4α in the kidney is not fully understood, but has been implicated in regulation of erythropoietin synthesis.

As in the liver, prenatal exposure to dexamethasone caused a significant increase (64% increase) in renal HNF4α expression. The increase in renal HNF4α mRNA was observed early (in one week old offspring) and persisted into adulthood. This was associated with significantly elevated levels of erythropoietin in circulation (110% increase). Moreover, animals that were exposed to prenatal dexamethasone had significantly increased red blood cell mass (7% increase), presumably as a result of upregulation of erythropoietin.

LIST OF ABBREVIATIONS (IN ALPHABETICAL ORDER)

ACE	angiotensin-converting enzyme
ACTH	adrenocorticotropic hormone
AT1	angiotensin II type 1 receptor
AT2	angiotensin II type 2 receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
11β-HSD1	11beta –Hydroxysteroid dehydrogenase type 1
11β-HSD2	11beta –Hydroxysteroid dehydrogenase type 2
Bp	blood pressure
CBG	corticosteroid binding globulin
CRH	corticotropin releasing hormone
EPO	erythropoietin
GR	glucocorticoid receptor
HNF	hepatocyte nuclear factors
HPA	hypothalamic-pituitary-adrenal
HIF	hypoxia-inducible factor
MR	mineralocorticoid receptor
Pck2	phosphoenolpyruvate carboxykinase 2
siRNA (or shRNA)	small (or short) interfering RNA

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CHAPTER 1

1.1

BACKGROUND

A series of worldwide epidemiological studies starting in the mid 1980s, have provided evidence for the strong association between disturbances in fetal development, as represented by low birth weight and cardiovascular mortality in adult life. The numerous accompanying animal and human studies that have followed since, have attempted to understand the myriad molecular, cellular, metabolic, neuroendocrine and physiological basis for understanding how adverse environmental effects, acting on the developing fetus, result in permanent alterations of the developmental pattern of cellular proliferation and differentiation in key tissue and organ systems, with resultant pathological consequences observed in adult life. (McMillen and Robinson 2005).

Our laboratory in Edinburgh, led by Professor Jonathan Seckl, as well as other investigators internationally, have hypothesized that excessive fetal glucocorticoid exposure may be an important mediator of low birth weight and later life chronic diseases such as hypertension and type 2 diabetes mellitus.

1.1.1

THE DEVELOPMENTAL ORIGINS OF DISEASE

That early life events play an important role in the later life phenotype is now well recognized. This concept is not without precedence; for instance, well over a century before, Weisman demonstrated that the early environmental temperature plays a critical role in phenotypic development in butterflies. Thus butterflies that hatched in summer were colored differently from those that hatched in winter, and this season-dependent coloration could be mimicked by incubating larvae at different temperatures.

Traditionally, cardiovascular disease and diabetes have been linked to adult lifestyle factors like diet, smoking and obesity. For example, in recent years, Willett, in 1994, noted that consumption of a diet high in fat, low in starch and fibre, appears to substantially increase the risk of cardiovascular mortality (Willett 1994). However, clinical epidemiological evidence suggests that prenatal experience may play an important role in the risk of development of subsequent cardiovascular and metabolic disease. Barker et al identified an association between the prevalence of cardiovascular disease among middle-aged men and low birth weight early in the 1980s (Barker, 1994). Their work indicated that adverse factors experienced in early life resulted either in death, or adaptative changes that might have contributed to disease risk in later adult life (Barker, 1994). Such findings were verified through large follow-up studies of patient cohorts across the United Kingdom, thus providing

evidence of associations between low birth weight and subsequent adult risk of cardiovascular disease, hypertension and diabetes. Since then, he and his colleagues, as well as other investigators, have demonstrated that low birth weight predicts the subsequent development of diseases like hypertension (Barker et al. 1990), ischemic heart disease , type 2 diabetes mellitus (Phillips et al. 1994), vascular dysfunction (Martyn et al. 1995), obesity (Yajnik 2000) and dyslipidemia (Barker et al. 1993c) in adult life (Barker et al. 2001). However it should be noted that the relationship between the risk of developing these chronic diseases and low birth weight is a continuous and quantitative one: the lower the birth weight, the greater the risk of developing later life hypertension. Additionally, the influence of birth weight on later disease is independent of the commonly acknowledged lifestyle risk factors in adulthood, namely adult weight, social economic status, smoking, alcohol consumption and sedentary lifestyle. Indeed, the adult 'lifestyle' risk factors are additive to the influence of early life (Barker 1990, Barker et al. 1993a, Law et al. 1995).

Further studies have led to a re-evaluation of the early work, demonstrating that subtle deviations in the fetal growth pattern leading to disproportion at birth (thinness, large head circumference in proportion to body length, small abdominal circumference, relative larger placenta when compared to birth weight) are more significant predictors of later disease risk (Langley-Evans 2001, Yajnik et al. 1995). In fact, in a British population, a baby with low birth weight and a large placenta had a relative risk of hypertension in adulthood that is three times higher than a baby with normal birth weight and a normal- sized placenta (Barker et al. 1990). For each birth weight, both systolic and diastolic pressures rose with placental weight, and for each placental weight both pressures fell with increasing birth weight. The highest pressures were among people who had been small babies with large placentas and the lowest were among people who had been large babies with small placentas (Barker et al. 1990)

Furthermore, the influence of early life on adult health is present in both developing countries, such as India (Yajnik et al. 1995, Krozowski et al. 1999, Stein et al. 1996) and Jamaica (Forrester et al. 1996), as well as the more developed countries like the UK, USA, Sweden and Australia.

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To explain the association between low birthweight and adult type 2 diabetes, Hales and Barkers proposed that in suboptimal fetal environment, the fetus makes an adaptive response to maximize uptake and conservation of any available nutrient, thus optimizing the growth of key body organs at the expense of others, leading to a permanent altered postnatal metabolism designed to enhance offspring postnatal survival under conditions of poor nutrition. (Hales and Barker 2001, Barker et al. 1993b, Hales and Barker 1992). This adaptation of the thrifty phenotype was thought to confer a survival advantage in these fetuses, arming them with the best ability to withstand any poor postnatal environment. It was proposed that these adaptations only became detrimental when nutrition was more abundant in the postnatal environment, than it had been in the prenatal environment.

This ability of the early environment to influence the phenotypic outcome of the adult individual is thought to reflect developmental plasticity, in which normal processes allow a range of phenotypes to develop from a single genotype (Gluckman et al. 2005a). The human fetus and infant can respond to unbalanced nutrition and other adverse influences by changing their developmental and growth trajectories (Gluckman et al. 2007, Gluckman and Hanson 2004). Plasticity may be responsible for the development of adaptive mechanisms that will ensure a successful survival later in life. (Bateson 2001, Bateson et al. 2004).

Cues for plasticity operate particularly during early development; they may affect a single organ or system, but generally they induce integrated adjustments in the mature phenotype, a process that is thought to be dependent on prediction of the

mature environment and may be underpinned by non-genetic e.g. epigenetic mechanisms. Thus, an adverse intrauterine environment results in responses that alter the developmental trajectory in preparation for harsh postnatal conditions. In generating a varied range of phenotypes adapted to thrive in different environments, developmental plasticity will contribute to the perpetuation of the genotype.

Unfortunately, developmental plasticity may also influence the eventual pathogenesis of chronic human disease by mismatch in the predicted versus actual postnatal environment. (Gluckman et al. 2008, Gluckman et al. 2005b, Gluckman et al. 2005d, Gluckman et al. 2005c, Gluckman et al. 2005a, Gluckman et al. 2007, Bateson et al. 2004). Mismatch between the anticipated and the actual mature environment would have adverse consequences on organism, and the greater the mismatch, the greater the risk of development of chronic diseases like high blood pressure, obesity and type 2 diabetes mellitus(Gluckman et al. 2007).

1.1.2

PROSPECTIVE EPIDEMIOLOGICAL EVIDENCE FOR LINK BETWEEN LOW BIRTH WEIGHT AND LATER DISEASE: DUTCH HUNGER FAMINE AND THE LENINGRAD SIEGE

The earliest epidemiological studies of fetal programming were retrospective studies focused on the association between birthweight and adult disease in geographically localized populations. Barker et al demonstrated a relationship between birthweight and adult diseases like hypertension, insulin resistance, vascular dysfunction, obesity and dyslipidemia. Barker and colleagues suggested that poor nutrition in pregnancy impairs fetal growth or promotes disproportionate fetal growth; leading to subsequent adaptations that promote survival in adverse conditions could lead to limited physiological function and disease in the long term.

The significance of maternal nutrition, the effect of poor nutrition on birthweight and subsequent adulthood disease was addressed in studies of exposure to famine, most notably the Dutch Famine of 1944–1945, which provides a powerful quasi-experimental prospective model to study the long-term consequences of maternal exposures in defined stages of gestation (Ravelli et al. 1998, Stein et al. 2006, Lumey et al. 1993, Ravelli, Stein and Susser 1976, Ravelli et al. 1999, Roseboom et al. 1999, Roseboom et al. 2001b, Roseboom et al. 2001a). The Dutch famine was at its worst for 5 months from December 1944 to April 1945, and this has provided data on the influence of maternal under-nutrition during different periods of gestation on later

offspring health (Morley 2006). For instance, data showed that the exposure to the Dutch famine (where average daily caloric intake was limited to 1680–3360 kJ) during late gestation was associated with increased adult obesity and glucose intolerance while famine exposure early in gestation resulted in hypertension (Stein et al. 2006).

In contrast, the one other study of adults exposed to famine during gestation, also during the second world war in Europe, conducted among survivors of the siege of Leningrad (now St. Petersburg) Russia, no association was observed between famine exposure and blood pressure levels at age 40 years (Stanner and Yudkin 2001). Data relating to the Leningrad siege, however, did not include birth weights, and the famine lasted much longer (almost 2 and a half years), so that most subjects would have been exposed throughout gestation and in infancy, and many mothers would have been exposed to famine before conception. As the nutritional status both before and after the famine periods in Leningrad was poor, the predictive adaptive responses may therefore have been appropriate for the postnatal environment experienced.

On the other hand, nutrition following the Dutch famine was relatively plentiful and the subsequent adult nutritional environment was much better than that predicted by the predictive adaptive responses, with subsequent manifestation of disease. It has been thus suggested that an important difference between subjects exposed to the Dutch versus Leningrad famines may be that the former experienced improved nutrition postnatally whereas most of the latter did not (Morley 2006).

Epidemiological evidence have also unraveled one other important aspect about the perinatal environment's effect on the risk of development of metabolic syndrome and cardiovascular disease: that impaired fetal growth followed by rapid catch-up in infancy might be an even stronger predictor of obesity, hypertension, non-insulin-dependent diabetes and coronary artery disease (Singhal A 2004, Langley-Evans 2006).

Data from other population-based studies suggest that growth restriction *in utero* is less detrimental to health in the long term when postnatal nutrition is compromised to a similar degree (Primatesta, Falaschetti and Poulter 2003). In keeping with the hypothesis of 'catch-up growth', babies that are born small but grow rapidly in the first months of life to eventually achieve normal centiles, have been identified as being at particularly risk of developing cardiovascular disease, and/or the metabolic syndrome later in life (Eriksson et al. 1999).

1.2

FETAL PROGRAMMING MODELS

To better understand the molecular mechanisms behind the epidemiological findings, animal models with shorter lifespan have been developed under controlled experimental conditions.

In the literature, many such experimental "programming" models (such as maternal malnutrition and/or protein restriction, uterine artery ligation, excessive fetal exposure to glucocorticoids, among others,) have reliably and consistently recapitulated the epidemiological observation of low birth weight offspring and the development of cardiometabolic perturbations (such as higher blood pressures and hyperglycemia), allowing for the dissection of molecular mechanisms that could explain the link between low birth weight and later life disease.

In essence, this experimentally useful notion of "programming" encapsulates the idea that a particular factor can act during a sensitive developmental period to reliably "program" consistent changes in the development and organization of specific tissues that are concurrently vulnerable, thus producing effects that persist throughout the adult life (Seckl 2004), which may then lead to a greater risk of chronic disease development. Due to different cells and tissues being sensitive at different times or different stages of development, the varying environmental stimuli

will exert distinct effects, depending on the nature of that stimulus as well as its timing (Seckl 2004).

A number of factors may have programming effects, but most available and consistent evidence relates to maternal nutrition and hormones, but other agents may include toxins and drugs (Csaba 1986). Among hormones, sex steroids and glucocorticoids are thought to be particularly important.

Perinatal programming by sex steroids is perhaps one of the best documented examples of programming. In many vertebrate species, male fetuses demonstrate a short burst of androgen secretion around the time of birth, which permanently programs steroid metabolising enzyme expression in the liver, as well as the size, connection and neurochemistry of specific hypothalamic nuclei, which ultimately affects sexual behaviour (Arai and Gorski 1968, Gustafsson et al. 1983). For instance, the sexually dimorphic nucleus of the preoptic hypothalamic area is larger in male rats because testosterone inhibits apoptosis in this locus specifically between postnatal days 6 and 10, resulting in the male adult phenotype (Davis, Popper and Gorski 1996). Similarly, the programming effects of oestrogens on the developing central nervous system is well-recognized (Simerly 2002). Despite being exerted only during specific perinatal periods, the organizational effects of sex steroids persist throughout life, largely unaffected by any subsequent sex steroid manipulations.

1.2.1

PROGRAMMING BY GLUCOCORTICOIDS

Glucocorticoid hormones play pivotal roles in both normal physiology and coordinating response to stress. In mammals, glucocorticoids regulate a variety of important cardiovascular, metabolic, immunological and other homoeostatic function. In particular, glucocorticoids play an important role in the regulation of blood pressure, glucose and stress. For instance, excessive glucocorticoid levels, resulting from either exogenous administration or endogenous overproduction (e.g. in Cushing's syndrome), with subsequent development of diabetes, hypertension (Baid and Nieman 2004, Thomson et al. 2007, Howlett, Rees and Besser 1985) and depression (Chrousos and Kino 2007, Welberg, Seckl and Holmes 2000)

Studies in our laboratory have used a well-validated animal model: the dexamethasone- programmed rat, whereby dexamethasone 100 mcg/kg is given during embryonic days 15 to 21 of maternal gestation in rats (third trimester). In this model, excessive fetal glucocorticoid (dexamethasone) exposure not only reduced offspring birthweight by about 10-14% consistently, but also predisposed adult offspring to higher blood pressures, glucose intolerance and hypercortisolism (Benediktsson et al. 1993, Lindsay et al. 1996, Nyirenda et al. 1998, Levitt 1996).

Dexamethasone is a poor substrate for 11β-HSD2 in the placenta, which is an efficient enzymic barrier that metabolize maternal active glucocorticoids into

inactive glucocorticoids, thereby keeping glucocorticoid levels in the fetal circulation much lower than in the maternal circulation. In this experimental model, prenatal administration of dexamethasone reduces birth weight, presumably by increasing fetal glucocorticoid load and its catabolic actions (as detailed in the section on glucocorticoid physiology) especially during the phase of greatest fetal somatic growth during the third trimester.

Similar programming effects in the offspring were also observed in rodent models using carbenoxolone, a potent inhibitor of placenta 11β-HSD2, allowing increased endogenous maternal glucocorticoid to pass through the placental enzymic barrier unmetabolised and thereby increasing fetal glucocorticoid load. These effects of carbenoxolone are independent of changes in maternal blood pressure or electrolytes, but do require the presence of maternal glucocorticoids: the offspring of adrenalectomised pregnant rats are protected from carbenoxolone effects upon birth weight or adult physiology (Lindsay et al. 1996).

This model of glucocorticoid programming of the fetus, as well as work from other investigators, has led to the hypothesis that fetal overexposure to glucocorticoids might mediate the link between low birth weight and risk of cardiovascular and metabolic diseases in adulthood (Seckl 1994, Nyirenda et al. 1998). Not surprisingly, prenatal glucocorticoids have been found to effect changes in corticosteroid biology, which in turn mediates the programming effects observed in the offspring.

For instance, in this model, GR was found to be upregulated in the periportal zone of the liver. This was associated with upregulation of PEPCK, a key gluconeogenic enzyme (known to be transcriptionally upregulated by GR) likely contributing to hyperglycemia observed (Nyirenda MJ et al 1998). Similarly, downregulation of GR in the hippocampus was observed leading to basal and stress hypercortisonemia (Levitt NS et al 1996), whereas upregulation of GR in the amygdala was associated with anxiety-like behaviour observed in the rat offspring (Welberg LAM et al 2001). Additionally, upregulation of hepatic 11β-HSD1, an important enzyme that amplifies local glucocorticoid action, has been observed in dexamethasone-programmed marmoset offspring that developed features of the metabolic syndrome (Nyirenda MJ et al 2009).

In the next sections, pertinent aspects of glucocorticoid biology will be discussed, given the important role of glucocorticoids and placental 11β-HSD2 in the developing fetus, as well as the changes in corticosteroid biology observed in this programming model.

1.2.1A

MOLECULAR MECHANISMS OF GLUCOCORTICOID ACTION

Glucocorticoids are produced by the adrenal glands and pass through the plasma membrane into the cytoplasm in target organs where they bind to specific glucocorticoid receptor (GR), a 94 kDa protein which is a member of the nuclear receptor superfamily of ligand-activated transcription factors (Hollenberg et al. 1985, Yamamoto 1985, Muller and Renkawitz 1991). In certain tissues, glucocorticoids can bind to and activate the mineralocorticoid receptor (MR). Within the cytoplasm, GR is bound to HSPs (heat shock proteins). Upon hormone binding, activated GR dissociates from heat shock proteins and forms a homodimer. These then translocated to the nucleus and bind to specific DNA-responsive elements (glucocorticoid response elements) to affect target gene transcription (Gronemeyer 1992, Beato, Herrlich and Schutz 1995). Glucocorticoids may also bind to receptors localized on the cell membrane and, through intracellular signaling pathways, mediate fast non-genomic action.

The GR has at least two isoforms (GR_α and GR_β). GR_β, a truncated form of GR_α, is an inactive species of GR. The molecular weights of these receptor isoforms are 97 and 94 kDa respectively. The GR_α resides mainly in the cell cytoplasm and represents the classic GR which functions as a ligand-dependent transcription factor. The GR_β, however, does not bind glucocorticoid agonists and exerts a dominant negative effect upon the transcriptional activity of GR_α (Chrousos 2004, Zhou and

Cidlowski 2005, Duma, Jewell and Cidlowski 2006, Kino et al. 2009, Oakley et al. 1999).It has been postulated that the relative proportion of GR_{α} and GR_{β}, not just total GR expression levels, might account for tissue-specific differences in GR activity.

Glucocorticoid action is controlled and modulated at several levels. First, secretion of glucocorticoids from the adrenal cortex is controlled by the hypothalamic-pituitary-adrenal (HPA) axis, via a classical negative feedback loop. Corticotrophin releasing hormone (CRH) synthesized in the neurons of the paraventricular nucleus (PVN) of the hypothalamus is released into the hypophyseal portal circulation. Corticotrophin releasing hormone stimulates adrenocorticotropic hormone (ACTH) synthesis and release from corticotroph cells of the anterior pituitary gland. In turn, adrenocorticotropic hormone stimulates production and release of glucocorticoids from the zona fasciculata-reticularis of the adrenal cortex. Glucocorticoids act on target organs throughout the body and provide negative feedback inhibition of CRH and ACTH in the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos 1995). Additionally, HPA axis activity can also be modulated by higher centres, notably the hippocampus and amygdala (Feldman and Conforti 1980, Jacobson and Sapolsky 1991).

After secretion from the adrenal cortex, the binding of circulatory glucocorticoids to plasma proteins such as corticosteroid binding globulin (CBG) and albumin may affect the circulatory glucocorticoids in 2 ways:

1. By limiting the concentration of free glucocorticoids (which are biologically active) and
2. Protecting the hormone against degradation by hepatic p-450 enzymes.

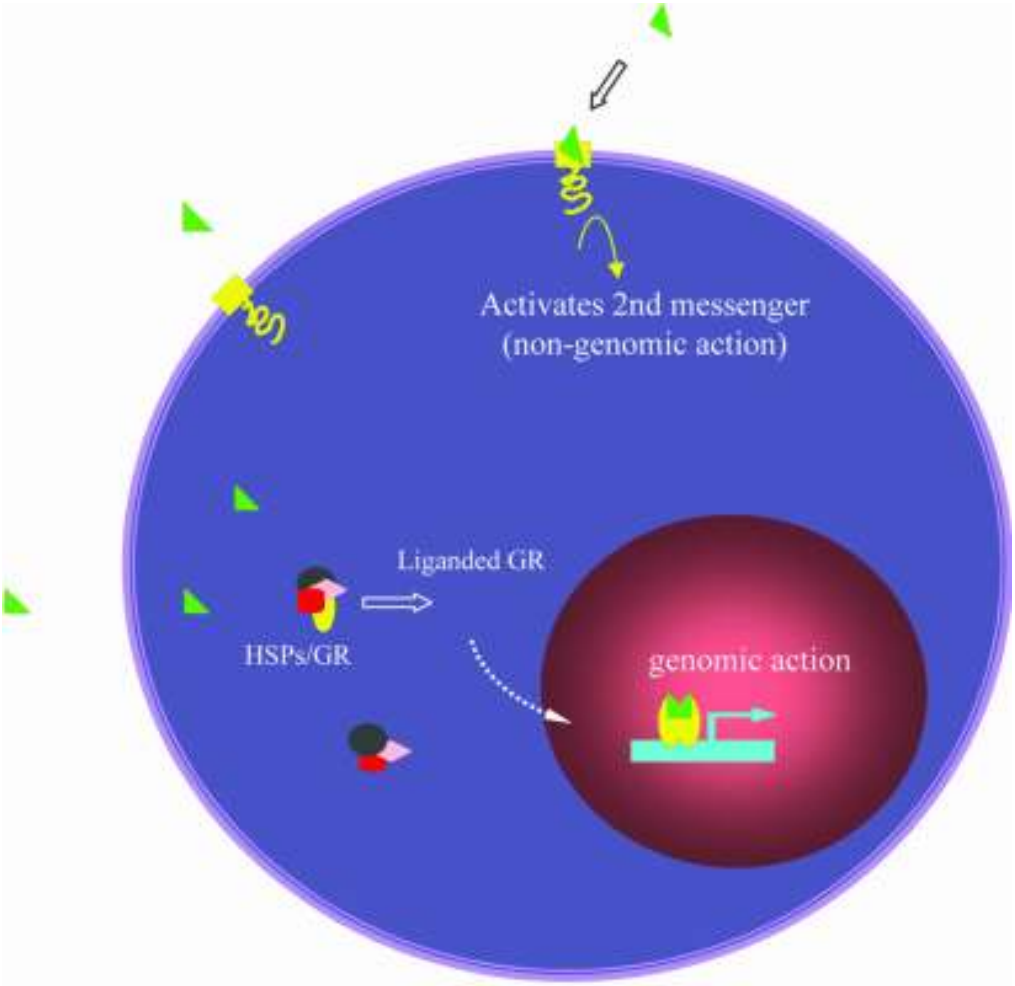

Fig 1-1

Schematic representation of action of glucocorticoid hormones

Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease (Drake, Tang and Nyirenda 2007)

CRH Corticotrophin releasing hormone

ACTH Adrenocorticotropic hormone

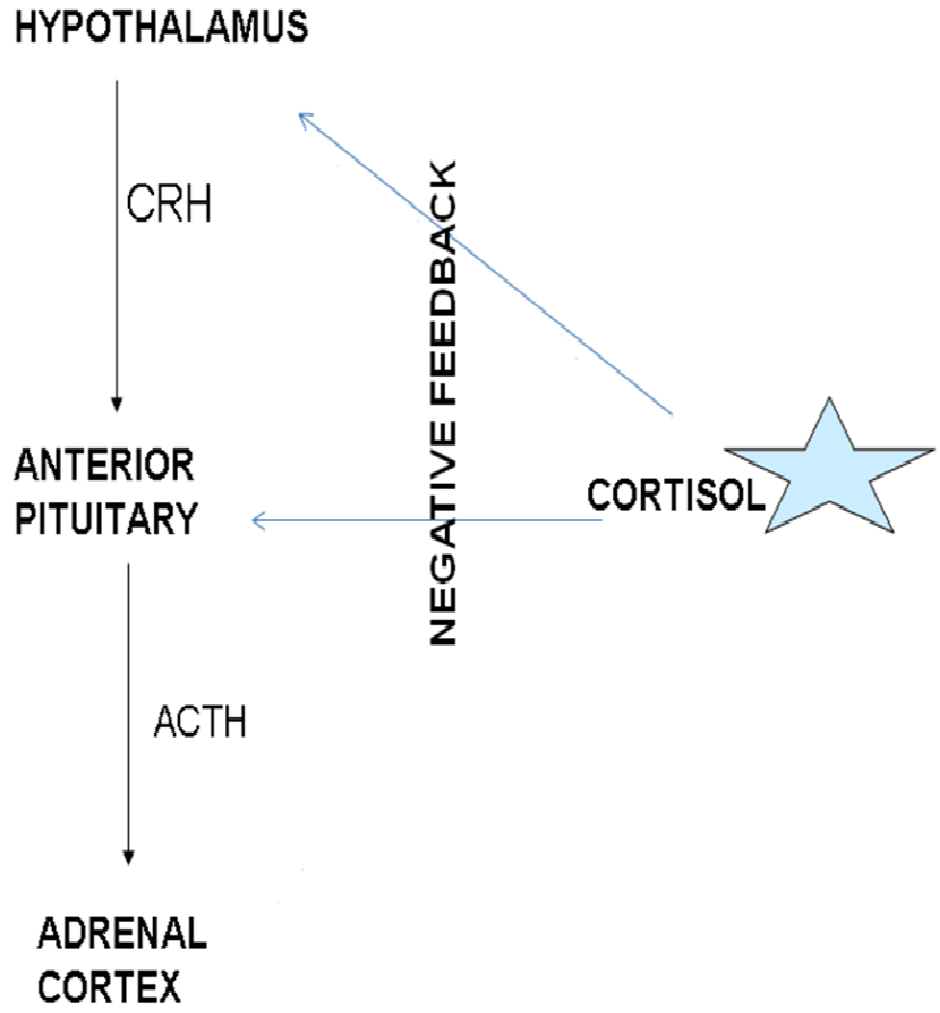

Fig 1-2 The hypothalamic-pituitary-adrenal (HPA) axis

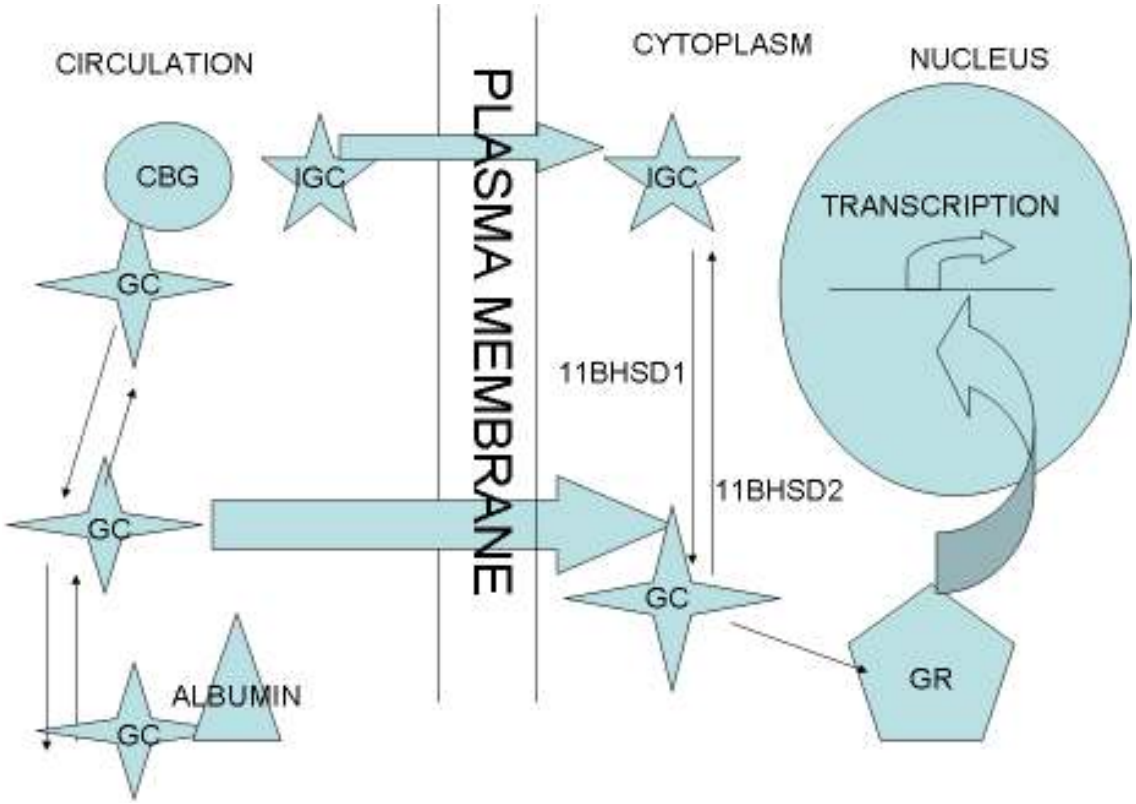

Fig 1-3 Determinants of glucocorticoid action

Legend

GC Glucocorticoid

IGC Inert form of Glucocorticoid

GR Glucocorticoid Receptor

CBG Corticosteroid Binding Globulin

11β-HSD1 11 Beta Hydroxysteroid Dehydrogenase type 1

11β-HSD2 11 Beta Hydroxysteroid Dehydrogenase type 2

At a cellular level, 2 important enzymes, 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes type 1 and 2 regulate local glucocorticoid concentrations by either catalyzing activation or inactivation of glucocorticoids. For example, although glucocorticoids and mineralocorticoids bind with equal affinity to MR ex-vivo (Krozowski and Funder 1983), it is only mineralocorticoids which transactivate MR in aldosterone –target tissues in vivo. This is accomplished, as discussed below, by 11β-HSD2, which co-localises with MR in the distal nephron, acting as an intracellular pre-receptor barrier, and selectively catalyses the rapid conversion of physiologically active glucocorticoids (cortisol in man, corticosterone in rats) into inactive 11-keto glucocorticoids (cortisone and 11-dehydrocorticosterone respectively) (Edwards et al. 1988, Funder et al. 1988). Thus, only mineralocorticoids activate MR in vivo.

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1.2.1B

GLUCOCORTICOID ACTION DURING DEVELOPMENT

GR is expressed in most fetal tissues from mid-gestation onwards (Schmid et al. 1995, Cole et al. 1995b) and in the placenta (Sun, Yang and Challis 1997), while expression of MR has a more limited tissue distribution and is present only at later gestational stages, at least in rodents (Brown et al. 1996). Glucocorticoids are important for the structural development of organs and functional maturation of many key fetal tissues in preparation for birth (Speirs, Seckl and Brown 2004) and extrauterine life (Cheng et al. 1980, Ballard 1989).

A number of studies have shown that significant maturational changes in organ systems, such as the lungs, heart, liver, gut and kidneys (Bian, Seidler and Slotkin 1993, Bian, Seidler and Slotkin 1992, Celsi et al. 1997), are glucocorticoid-dependent, and can be induced prematurely by exogenous glucocorticoid administration (Ballard 1979, Fowden 1995), underpinning their widespread therapeutic use in threatened preterm labour and in the perinatal period, particularly to accelerate the rate of lung maturation (Ward 1994). Furthermore, GR-null mice die within the first few hours after birth of respiratory failure, due to severe lung atelectasis, and have severely retarded maturation of the adrenergic chromaffin cells and hepatic gluconeogenic enzymes (Cole et al. 1995a).

However, there is also evidence that supraphysiological concentrations of glucocorticoids are harmful to the fetus. For example exogenous administration of glucocorticoids has been shown to retard fetal growth and produce lowered weight at birth in humans, non-human primates and other experimental mammals (Reinisch et al. 1978, Novy and Walsh 1983, Mosier et al. 1982, Berry et al. 1997, Nyirenda et al. 1998, Seckl 1994). Furthermore, cortisol levels are increased in human fetuses with intrauterine growth retardation (Goland et al. 1993, Goland, Conwell and Jozak 1995) or in pregnancies complicated by pre-eclampsia, which may indicate a role for endogenous cortisol in fetal growth retardation (Goland et al. 1993, Goland et al. 1995).

There may be significant differences between the effects of fetal exposure to endogenous glucocorticoids and those of synthetic glucocorticoids relevant to programming models. For example, although endogenous glucocorticoids can bind both GR and MR and the effects in tissues such as brain may be mediated by both of these receptors, synthetic glucocorticoids are more selective for MR (Drake, Tang and Nyirenda 2007). Similarly, there may be differences in local glucocorticoid concentrations in tissues, which may be governed by differences in transport (e.g. across the blood–brain barrier) and metabolism of endogenous and synthetic glucocorticoids (McCabe et al. 2001, Rashid and Lewis 2005).

1.2.1C

11β-HYDROXYSTEROID DEHYDROGENASE ENZYMES

Beyond the classical modulations of circulatory glucocorticoid levels, intracellular glucocorticoid activity in the peripheral tissues is determined by the 11β-hydroxysteroid dehydrogenase enzymes; type 1 (11β-HSD1) and type 2 (11β-HSD2). The type 1 isozyme (11β-HSD1) and type 2 isozyme (11β- HSD2) are distinct enzymes.

11β-HSD1 is made up of a 34kDa glycoprotein consisting 187 amino acids (in rats) and 292 amino acids (in humans) (Lakshmi and Monder 1988). It is located predominantly in the liver, fat, lung, brain and testis (Stewart and Krozowski 1999, Monder and Lakshmi 1989). In vivo, 11β –HSD1 acts primarily as a reductase, using NADP(H) as a cofactor, converting cortisone to cortisol (Jamieson et al. 1995), thereby amplifying intracellular glucocorticoid action.

Up-regulation of 11β-HSD1 may be an important key mechanism in amplifying the local glucocorticoid activity in key metabolic tissues such as visceral fat and/ or liver, which may also contribute to important pathophysiological processes (Seckl and Walker 2001). Indeed, in transgenic models, animals with selective over expression of 11β-HSD1 in visceral fat or in the liver showed features of increased gluconeogenesis and the metabolic syndrome, mediated by greater pro-inflammatory cytokines delivered to the hepatocyte in the portal circulation (Masuzaki et al. 2001).

Indeed, in marmosets at least, prenatal glucocorticoid treatment resulted in upregulation of hepatic 11β-HSD1 mRNA, possibly responsible for the later development of adult hyperglycemia observed in this model (Nyirenda 2009).

On the other hand, 11β-HSD2 catalyses the reverse reaction: converting cortisol to cortisone. The human 11β-HSD2 is 41 kDa, 405 amino acids long, requires NAD^+ as a cofactor and has a lower Km (2-10 nM) for steroid substrate. 11β-HSD2 is co-localized with mineralocorticoid receptor, and is present in the kidney and placental microsomes in sheep and humans (Narayfejestoth, Watlington and Fejestoth 1991, Agarwal et al. 1994, Albiston et al. 1994) .

In the kidney, 11β-HSD2 plays an important role in regulation of sodium reabsorption and blood pressure. By acting as a pre-receptor barrier, renal cellular 11β-HSD2 prevents glucocorticoids from activating MR. Inactivating mutations of the 11β-HSD2 gene allow illicit activation of MR by glucocorticoids, resulting in the syndrome of apparent mineralocorticoid excess (SAME)(Stewart et al. 1988), characterized by increased sodium reabsorption, hypertension and hypokalemia. 11β-HSD2 and other genes involved in renal glucocorticoid/ mineralocorticoid action are therefore important potential targets in programming of hypertension.

11β-HSD2 also plays a critical role in the placenta where it limits glucocorticoid access to the fetus. Concentrations of the active glucocorticoid cortisol are high in maternal blood during pregnancy. Although lipophilic steroids can pass through the feto-placental barrier, the presence of placental 11β-HSD2 results in the rapid

inactivation of cortisol to inert cortisone, thus minimizing foetal exposure (Campbell and Murphy 1977, Seckl 1997). Indeed, reduced placental activity of 11β–HSD2 was associated with lower birth weight in human infants (Kajantie 2003). Also, in regression analyses of AME patients with published genotypes, a lower birth weight was correlated with lower levels of enzyme activity (Nunez 1999), likely reflecting the result of increased glucocorticoid exposure to the developing infant.

In support of the glucocorticoid programming hypothesis, prenatal treatment with the synthetic glucocorticoid dexamethasone (which bypasses the placenta barrier unmetabolised) or carbenoxolone (a licorice derivative with potent placental 11β-HSD2 inhibitor activity) has been shown to increase offspring blood pressure, glucose and glucocorticoid levels in a number of species including non-human primates (Drake, Tang and Nyirenda 2007). The molecular mechanism that underpins glucocorticoid programming remains incompletely understood, but may involve coordinated alterations in glucocorticoid activity, through changes in receptor density and/or expression of enzymes that modulate local glucocorticoid concentrations, possibly involving the 11β-HSD enzymes in the different tissues.

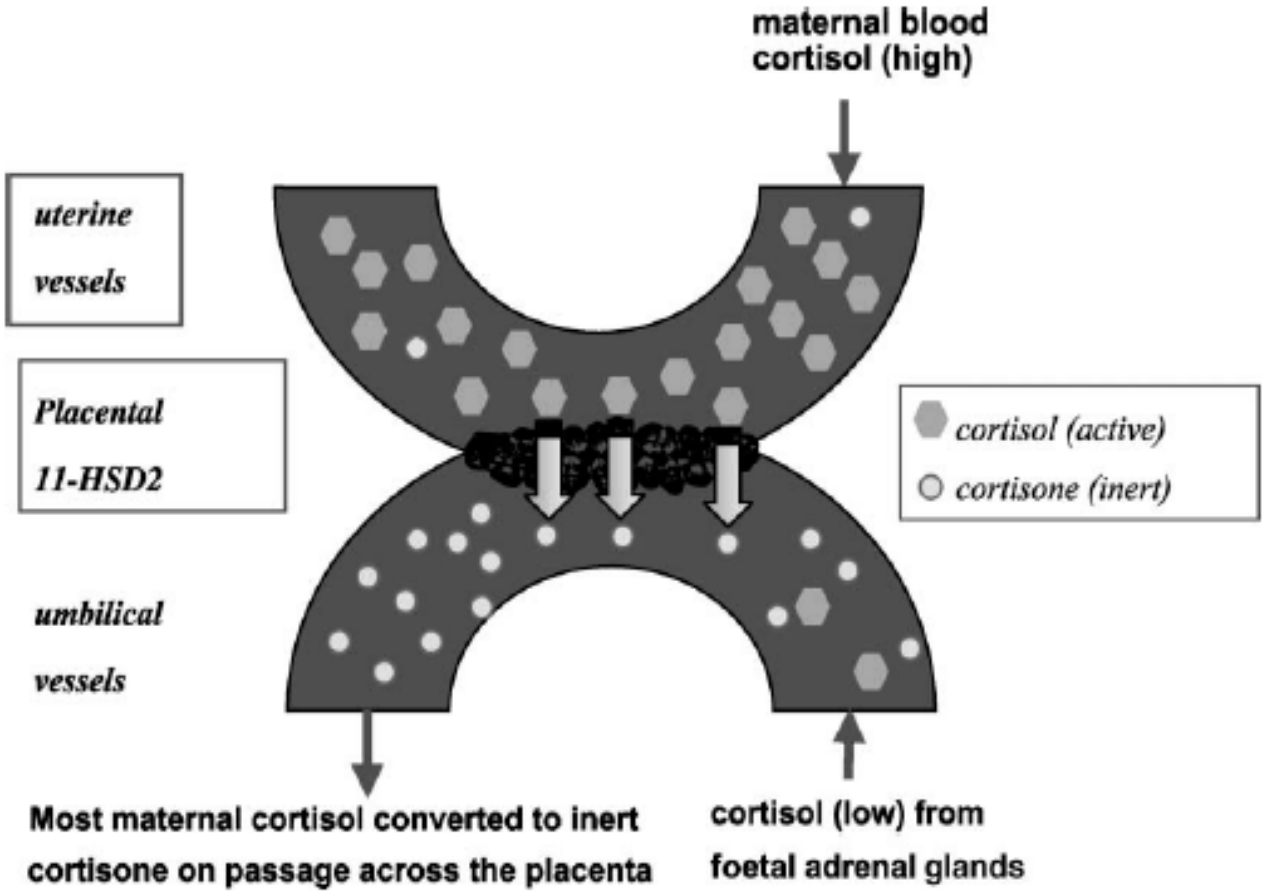


Fig 1-4 11β-HSD2 as placental barrier

1.3

PROGRAMMING OF HYPERTENSION

The programming of hypertension has been well-established by the dexamethasone rat model. Initial studies from our laboratory performed on rodents confirmed the hypothesis that excessive glucocorticoid exposure not only reduced offspring birthweight, but also predisposed adult offspring to higher blood pressures, glucose intolerance and hypercortisolism (Benediktsson et al. 1993, Lindsay et al. 1996, Nyirenda et al. 1998, Levitt 1996).These observations were observed in models using dexamethasone and carbenoxolone, a potent inhibitor of placenta 11β-HSD2, allowing increased endogenous maternal glucocorticoid to pass through the placental enzymic barrier. Dexamethasone is a poor substrate for 11β-HSD2, and in experimental models, prenatal administration of dexamethasone reduces birth weight, presumably by increasing fetal glucocorticoid load.

A similar effect is seen when the pregnant rats are treated with carbenoxolone. These effects of carbenoxolone are independent of changes in maternal blood pressure or electrolytes, but do require the presence of maternal glucocorticoids: the offspring of adrenalectomised pregnant rats are protected from carbenoxolone effects upon birth weight or adult physiology (Lindsay et al. 1996). Both male and female adult offspring of dexamethasone-treated pregnancies have elevated blood pressure (Benediktsson et al. 1993, Woods and Weeks 2005, Sugden et al. 2001, Levitt et al.

1996), although the mechanisms in the induction of hypertension in these models may be gender-specific (O'Regan 2004, McMullen 2005)

These observations have now been replicated in other laboratories, using other mammalian species, notably sheep and monkeys. Cortisol infusion into the foetus in utero elevates blood pressure in sheep (Tangalakis et al. 1992, Kari et al. 1994). Excess cortisol also directly elevates blood pressure at birth in humans (Kari et al. 1994) and sheep (Berry et al. 1997). However, programming to occur, such effects need to persist. Of note, adult hypertension is produced in sheep exposed to excess glucocorticoid in utero, either in the form of maternally administered dexamethasone or cortisol (Dodic et al. 1998, Dodic et al. 2002b, Dodic et al. 2002a, Dodic et al. 2001b).

The timing of glucocorticoid exposure appears to be important. Exposure to glucocorticoids during the final week of pregnancy in the rat is sufficient to produce permanent adult hypertension (Levitt et al. 1996). Wintour and others, however have found that the sensitive window for programming hypertension in dexamethasone-treated sheep is in early gestation, in the early stages of nephrogenesis (Wintour 2003, Dodic et al. 1998, Gatford et al. 2000, Moss et al. 2001). Such differences may be primarily due to the complex species-specific patterns of expression of glucocorticoid receptor, mineralocorticoid receptor and the isoenzymes of 11β-HSDs, which regulate maternal glucocorticoid transfer to the foetus and modulate glucocorticoid action in individual tissues (Seckl 1997).

In this respect this late gestational timing is similar to human evidence on the effect of famine during pregnancy on later metabolic disease in the offspring, suggesting that glucocorticoids may be involved in human programming of chronic diseases. Birth weight reduction is most significant when glucocorticoids are administered in the latter stages of pregnancy, an acknowledged period of maximum fetal somatic growth (Nyirenda et al. 1998). In humans, fetal blood cortisol levels are increased in intrauterine growth retardation, thus implicating endogenous cortisol in retarded fetal growth (Goland et al. 1993, Goland et al. 1995).

In our dexamethasone-programmed model, most investigators have found an increase of 10 mmHg of systolic blood pressure when measured using the tail-cuff method in adult rats (experiments were carried out on adult offspring between 4 months to 9 months old). (Benediktsson et al. 1993, Levitt NS et al 1996, O'Regan et al 2004). However, using sophisticated blood pressure measurements with radiotelemetry, which is unaffected by stress artefacts and therefore represents the gold standard in rodent blood pressure measurement, it was found that adult male offspring paradoxically had lower basal blood pressures in adulthood (4–8 mmHg lower); with the commonly expected hypertensive phenotype only being noted when these offspring were subjected to even mild disturbance or a more severe stressor (up to 30 mmHg higher than controls). They also observed that the isolated mesenteric vasculature dexamethasone-treated offspring had a greater sensitivity to noradrenaline and other vasoconstrictors and concluded that altered sympathetic responses mediate the stress induced hypertension in this model.

This finding highlights the importance of the blood measurement technique in assessing blood pressure, as rats are usually "stressed" and restrained during tail-cuff measurements. Indeed, dexamethasone-programmed show a greater increase in basal and stress related corticosterone (Levitt NS et al 1996), a finding that has been replicated in human studies, as further discussed in the next section. This raises the interesting possibility that the raised blood pressure observed in these animals using the tail-cuff method could also be related to the greater corticosterone levels induced by stress during restraint. O'Regan also observed a longer duration of blood pressure increases after minor "stressors", e.g. by an observer entering the room. This could mean that longer-term mechanisms, perhaps mediated by corticosterone on other regulatory blood pressure systems like the kidney, may be involved in this phenomenon, rather than the shorter term influences of the sympathetic nervous system.

In light of this finding (of hypotension in the "non-stressed" state as measured by radiotelemetry), there also remains the alternative possibility that increased mineralocorticoid activity with increased renal sodium reabsorption may be a compensatory response to the lower blood pressure observed (as per Guyton's observations that a decrease in blood pressure increases sodium retention). Thus, it would be interesting to further explore this issue and investigate if changes in mineralocorticoid signalling and downstream sodium retention, either as a compensatory mechanism in response to the hypotension observed in these adult animals, or as a primary change that allows corticosterone to initiate longer-term changes in blood pressure (or possibly both). This could be addressed experimentally

(e.g. by measuring if changes in mineralocorticoid signalling occur early in life before blood pressure changes, to determine if these changes are primary or secondary).

Dexamethasone programming likely produces an integrated adaptive response that lead to hypertension. Although the mechanisms of hypertension remains incompletely understood, changes in basal and stress hypercortisonemia (Levitt NS 1996), an increased sympathetic response to stress (O'Regan D, 2008) and insulin resistance (Nyirenda MJ 1998) have previously been observed in our model. Both male and female adult offspring of dexamethasone-treated pregnancies have elevated blood pressure (Benediktsson et al. 1993, Woods and Weeks 2005, Sugden et al. 2001, Levitt et al. 1996), although the mechanisms in the induction of hypertension in these models may be gender-specific (O'Regan 2004, McMullen 2005)

Michel Baum and colleagues at the University of Texas, Southwestern Medical Center have used a similar model of dexamethasone programming. However in their protocol, they used 200 mcg/kg of dexamethasone injected peritoneally daily from embryonic days 15 to 21. They have found increased proximal tubular type 3 Na^+/H^+ exchanger (NHE3), Na^+-K^+-$2Cl^-$ cotransporter (NKCC2), and Na^+-Cl^- cotransporter (NCC), but not α-, β-, and γ-epithelial Na^+ channel (ENaC) protein abundance compared with controls. This was associated with increased proximal sodium reabsorption. Additionally, the kidneys of dexamethasone programmed offspring had a 20% lower number of glomeruli. In their programmed offspring, higher blood pressures were noticed at 8 weeks. When renal denervation was

performed in young offspring, no hypertension developed in adult life and the changes in NHE3, NKCC2, and NCC were not observed (Baum et al 1993, Dagan et al, 2008).

Various other fetal programming models have been used to dissect the mechanisms of hypertension. These models include global food restriction or protein restriction (maternal malnutrition) during gestation (which is the best studied model), chronic hypoxia administered to the mother during the gestational period and placental insufficiency. Modes of dietary manipulation include global caloric restriction, dietary protein reduction, iron restriction or dietary fat supplementation. These have been extensively studied in both rodent and ovine models. In most rodent models of maternal nutrient restriction, dietary manipulation is carried out until weaning when the offspring go onto standard laboratory chow.

Offspring of treated mothers are generally born with low birthweight. In some of the models, there has been observed a congenital deficit in nephron number. Reduction in nephron numbers would predispose the individual to reduced renal sodium excretion and subsequent increased susceptibility to essential hypertension, particularly in the setting of dietary sodium excess (Brenner, Garcia and Anderson 1988). The exact mechanism of the reduction in nephron number has not been established, but several hypotheses have been proposed, e.g. changes in DNA methylation.

These animal models have also demonstrated that prenatal dietary manipulation cause reduced expression of components of the renin-angiotensin system during active nephrogenesis (Guron and Friberg 2000) that result in lower nephron numbers and hypertension in later life (Vehaskari et al. 2004, Woods et al. 2001).

Furthermore, other studies suggest an interaction between low-protein programming, glucocorticoids and the intrarenal renin-angiotensin system, such that an excess of glucocorticoids seen in these models can be a negative regulator of the components of the renin-angiotensin system in the fetus (Celsi et al. 1998, Segar et al. 1995). Since it has also been shown that angiotensin II can stimulate the expression of Pax-2 through angiotensin II type 2 receptor, negative regulation of intrarenal RAS could affect nephrogenesis and kidney development (Zhang, Moini and Ingelfinger 2004).

Other mechanisms that have been suggested to contribute to programming of hypertension include permanent alterations in the activity of the renin-angiotensin system (Bogdarina 2006), renal 11β-HSD2 (Bertram et al 2003,, Baserga et al 2010), components of the cardiovascular system altered vascular function, and increased expression of the angiotensin II type 1 and 2 receptors and angiotensin-converting enzyme (McMillen and Robinson 2005),. There might also be altered vascular responsiveness to other vasoconstrictors, with enhancement of endothelin-induced vasoconstriction and attenuation of endothelium-dependent vasorelaxation in sheep (Molnar et al. 2002, Molnar et al. 2003).

Intriguingly, glucocorticoids (as stress hormones) have been suggested as a possible candidate mechanism that underlies these various models of programming where the pregnancy has been obviously subjected to stress. For instance, protein restriction in maternal rats was associated with higher maternal cortisol levels and increased fetal glucocorticoid load (Langley-Evans 1996). The programming effect (on hypertension) in the protein restriction model was subsequently abolished when maternal glucocorticoid synthesis was inhibited by metyrapone, an inhibitor of maternal glucocorticoid synthesis (Langley-Evans 1997), indicating that glucocorticoids may play a part in the programming effects at least in the protein restriction model of fetal programming.

1.3.1

EVIDENCE FOR FETAL PROGRAMMING OF HYPERTENSION BY GLUCOCORTICOIDS IN HUMANS AND ITS RELEVANCE

The effects of prenatal glucocorticoid exposure observed in animal models could have huge implications if extrapolated to the human fetus. Glucocorticoids are given to pregnant women for various conditions, but robust data on long term disease in offspring are lacking. But, as discussed below, some evidence suggests neurobehavioral and blood pressure changes detected as early as childhood, highlighting the importance to better understand the underlying mechanisms glucocorticoid fetal programming.

The long-term effects of fetal glucocorticoid exposure in humans have been poorly investigated, mainly because these studies have been small and the duration of follow-up short. Studies aimed at establishing the long-term neurological and developmental effects of antenatal glucocorticoid exposure have been complicated by the fact that most of the children studied were born before term and were therefore already at risk of delayed neurological development.

Glucocorticoids are used as immunosuppressants to control various maternal conditions, such as connective tissue disorders (Rayburn 1992), and are used extensively in obstetric practice, primarily to accelerate lung maturation in cases of threatened preterm labour (Crowley, Chalmers and Keirse 1990), which may occur in

up to 10% of pregnancies. There is no doubt that synthetic glucocorticoids enhance lung maturation and reduce mortality in preterm infants. Additionally, a single course of prenatal corticosteroid is associated with a significant reduction in the incidence of intraventricular hemorrhage and a trend toward less neurodevelopmental disability (Crowley 2000) . However, 98% of British obstetric departments have prescribed repeated courses of antenatal glucocorticoids (Brocklehurst et al. 1999), despite little evidence for the safety and efficacy of such a regime (Whitelaw and Thoresen 2000). Recent data have suggested that a short antenatal glucocorticoid exposure in preterm babies is associated with increased blood pressure at 14 years (Doyle et al. 2000).

Yet another group of women - those at risk of bearing fetuses with congenital adrenal hyperplasia often receive low-dose dexamethasone from the first trimester to suppress fetal adrenal androgen overproduction (Carlson et al. 1999, Forest, Betuel and David 1989). Birth weight in such infants, however, has been reported as normal. Nevertheless, programming effects of antenatal glucocorticoids are seen in animal models in the absence of any reduction in birth weight (Forest, David and Morel 1993, Mercado et al. 1995). Hence, careful long-term studies of this group of children are warranted for the presence or absence of programming effects.

On the basis of findings in the prenatal dexamethasone-exposed rat model of low birth weight and adult hypercorticosteronemia with hypertension (Levitt NS et al 1996), studies have examined the relationship between birth weight and hypothalamic-pituitary-adrenal axis function in adult humans. As in other animals,

the human hypothalamic-pituitary-adrenal axis appears to be programmed by the early life environment.

Programming of the hypothalamic-pituitary-adrenal axis appears to occur in disparate populations (Phillips et al. 2000) and may precede overt adult disease (Levitt et al. 2000), at least in a socially disadvantaged South African population. Higher plasma and urinary glucocorticoid levels are found in children and adults born with lower birth weight (Clark et al. 1996, Phillips et al. 1998). In children, low birth weight is associated with altered adrenocortical responses to stress in boys and altered basal adrenocortical activity in girls (Jones et al. 2006). In adulthood, hypothalamic-pituitary-adrenal axis responses to corticotrophin stimulation is exaggerated in offspring with low birth weight (Levitt et al. 2000, Reynolds et al. 2001), reflecting the stress axis biology elucidated in animal models. Furthermore, this hypothalamic-pituitary-adrenal axis activation is associated with higher blood pressure, insulin resistance, glucose intolerance and hyperlipidemia (Reynolds et al. 2001).

1.4

DETERMINANTS OF BLOOD PRESSURE: THE IMPORTANT ROLE OF THE KIDNEY

Blood pressure must be tightly regulated to permit uninterrupted perfusion of all vital organs. For example, even transient interruption in blood flow to the brain will cause loss of consciousness, and longer interruptions will result in cellular hypoxia of unperfused tissues. Conversely, higher pressures that deliver flow exceeding metabolic demand provide little or no metabolic gain, but increase damage to blood vessels and organs. These considerations dictate the normal ranges of blood pressure (Lifton 2001).

A wide variety of physiologic systems that have pleiotropic effects and interact in complex fashion have been found to influence blood pressure. Amongst these include baroreceptors that sense acute changes in pressure in vessels; natriuretic peptides produced by the brain and heart in response to increased pressure in these organs; the renin-angiotensin-aldosterone system, which influences vascular volume homeostasis and vascular tone; the kinin-kallikrein system, which affects vascular tone and renal salt handling; the adrenergic receptor system, which influences heart rate, cardiac contraction, and vascular tone; and factors produced by blood vessels that cause vasodilation, such as nitric oxide, or contraction, such as endothelin. These

systems act in an integrated fashion to ensure adequate perfusion of all tissues despite widely varying metabolic demand (Lifton 2001).

Nevertheless, the kidney appears to be the key organ in the development of hypertension (Zandi-Nejad, Luyckx and Brenner 2006). Factors intrinsic to the kidney itself affect blood pressure, as demonstrated in renal transplantation (both in humans and animals), where blood pressure in the recipient after transplantation is related to the hypertension risk factors of the donor; that is, hypertension "follows" the kidney (Rettig et al. 1990, Guidi et al. 1996).

The relationship between renal sodium handling, intravascular fluid volume homeostasis, and hypertension, described initially by Guyton et al (Guyton et al. 1972) using systems biology, is well accepted. Guyton thus argued that convincingly that for chronic high blood pressure to persist, a defect in renal sodium excretion and pressure-natriuresis likely is present.

Genetic approaches to this disease have also begun to delineate molecular pathways underlying blood pressure, defining disease pathogenesis and identifying targets for therapeutic intervention (Lifton, Gharavi and Geller 2001). That single genes can impart large effects on blood pressure is demonstrated by rare Mendelian forms of high and low blood pressure (Lifton, 1996). Molecular genetic studies have now identified the exact mutations in 8 genes that cause Mendelian forms of hypertension and 9 genes that cause Mendelian forms of hypotension in humans. Intriguingly, all

of these genes are involved in renal salt and water homeostasis in the aldosterone-sensitive distal tubules and collecting duct in the kidney.

Physiologically, blood pressure determination follows Ohm's law (blood pressure equals to cardiac output multiplied vascular resistance). Increased salt and water reabsorption causes increase in cardiac output (Frank-Starling's law) initially. Autoregulation occurs after the initial phase such that the cardiac output normalizes, whilst blood pressure is maintained by an increased vascular resistance.

The kidney, via the production of erythropoietin, might also potentially affect blood pressure through changes in blood viscosity, in accordance with Poiseuille's law (below) where in a cylindrical vessel with laminar flow, blood pressure P is proportional its length (l), viscosity of the fluid (μ) and cardiac otput (Q), and inversely proportional to its radius to the 4th power (r^4)

$$P=\frac{8\mu LQ}{\Pi r^4}$$

It has been previously observed that prenatal dexamethasone exposure induced a prompt rise in foetal hepatic HNF4α mRNA. This increase occurred predominantly in the periportal region of the hepatic acinus and persisted into adulthood, congruent with the change in PCK2 mRNA (Nyirenda et al. 2006). This upregulation of PCK2, a rate-limiting enzyme of gluconeogenesis, probably contributed to the impaired glucose tolerance observed in this rodent model of dexamethasone programming.

The interaction between glucocorticoids and HNF4α is further supported by the recent identification in mouse HNF4α of an enhancer element containing a glucocorticoid response sequence (Bailly et al. 2001). Intriguingly, hypoxic regulation of EPO requires HNF4α, which is constitutively expressed in the major sites of EPO production: the fetal liver and the adult kidney. HNF4 binds to the EPO 3' enhancer adjacent to the HIF (hypoxia-inducible factors)-binding site (Ebert and Bunn 1999, Mietussnyder et al. 1992), interacting with HIFs to attract other co-activators of EPO. We therefore speculated that HNF4α might be upregulated in the kidney, together with increased expression of EPO.

1.5

HYPOTHESES

(Please see figure)

1. That the programming of hypertension following prenatal exposure to dexamethasone is mediated by gene expression changes in renal corticosteroid and/or mineralocorticoid signalling pathways, leading to impaired sodium excretion in the kidneys, progressing to later life hypertension and salt sensitivity.

2. That prenatal exposure to dexamethasone increases renal production of erythropoietin leading to increases in plasma hematocrit and viscosity leading to hypertension.

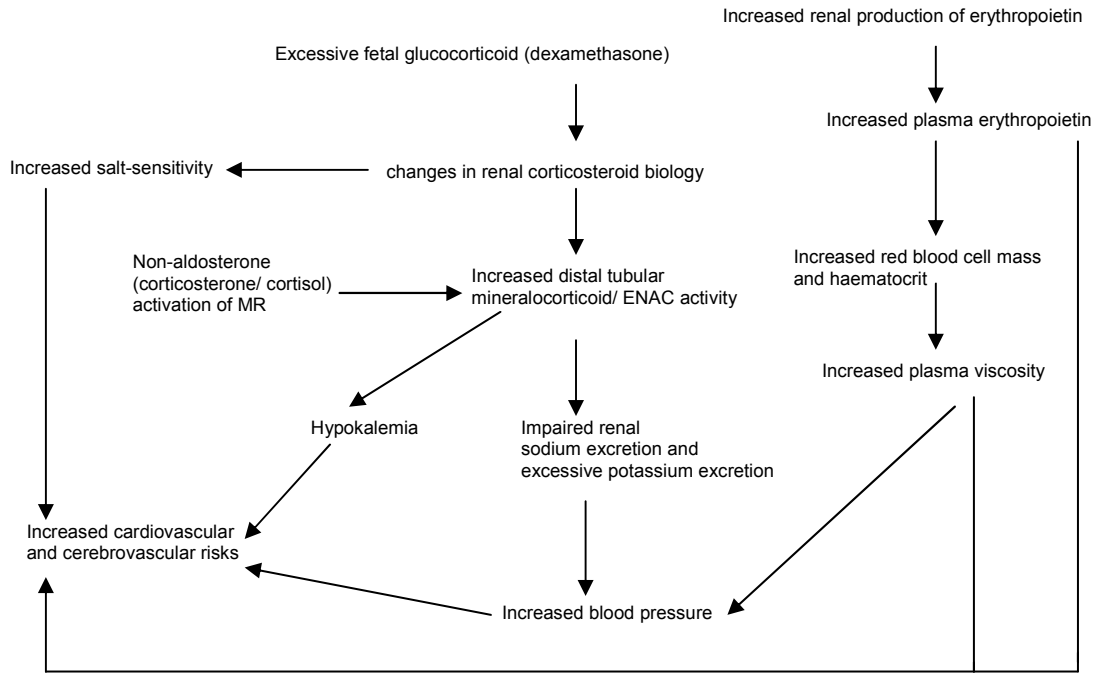


Figure 1-5 Proposed hypothesis and mechanisms by which excessive glucocorticoid (dexamethasone) might cause hypertension, increased salt-sensitivity and ultimately increased cardiovascular risk through key alterations in the kidney- namely, renal distal tubular 11βHSD2 activity and the renal production of erythropoietin.

CHAPTER 2

MATERIALS AND METHODS

2.1

ANIMALS

All animal experiments were carried out under a UK Home Office Licence. Male and female Wistar rats (200-250g) were bought from Charles River Laboratories, Essex, UK, and maintained under conditions of controlled lighting (lights on from 0700hrs to 1900hrs) and temperature (22ºC) and allowed free access to food standard rat chow (Special Diet Services, Essex, UK) and tap water.

After a week of settling in at the animal lab, rats were time-mated. Mating was confirmed by the identification of a vaginal plug; the day of plugging was taken as day 0 of pregnancy.

2.2

PRENATAL DEXAMETHASONE TREATMENT

Time-mated rats were given either subcutaneous dexamethasone (100µg per kg^{-} per day dissolved in 4% ethanol/ 0.9% saline at a final concentration of 200µg/ml) or vehicle (4% ethanol/ 0.9% saline) during the last week (day 15 to 21) of pregnancy. At least 5 pregnant rats were used per group in each experiment. At birth, the offspring were sexed and weighed and culled to eight pups per litter. This cohort of rat offspring was used for experiments done at 9 months of age. A second distinct group of male offspring were bred for experiments performed at E21, 1 week old (real-time PCR experiments) and 2 months old (plasma electrolytes and salt-sensitivity experiments). This second group of male offspring was culled to 6 male offspring per litter.

Only male offspring, selected at random from each litter, were used in all subsequent experiments.

The number of male offspring used for tail-cuff measurements was 9 male offspring per group. 6 male offspring per group were used for the real-time experiments. The number of rats used was based on estimations for the minimal sample size as detailed below (section 2.11).

The pups were thereafter weaned on postnatal day 21, and housed in cages 2-4 male offspring each cage.

The offspring were killed by decapitation in either the early postnatal period (1 week old) or in adulthood (9 month old) to collect samples. All animals were sacrificed in the fed state and samples quickly frozen in liquid nitrogen and stored at -70ºC for subsequent analyses.

In a separate cohort of animals, fetal livers from treatment and control groups were collected at embryonic day E21 for erythropoietin studies.

2.3

BLOOD PRESSURE MEASUREMENTS

In rodents blood pressure can be measured by a number of methods. The most accurate method is direct blood pressure measurement. This is an invasive surgical procedure (where blood pressure typically obtained on the rodent's carotid artery) that is used as the gold standard to compare the accuracy of non-invasive technologies (Bunag 1973). The disadvantages of direct blood pressure methods like radiotelemetry include morbidity associated with the initial surgical procedure, a potential increase in the animal's stress, high costs of the equipment set-up and maintenance, as well as the financial, equipment and human resources relating to surgery and anaesthesia needed for intra-arterial cannulation.

The non-invasive methods of blood pressure involve utilizing a tail cuff placed on the rodent's tail to occlude the blood flow. Upon deflation, one of several types of non-invasive blood pressure sensors, placed distal to the occlusion cuff, can be used to monitor the blood pressure.

In our studies we employed photoplethysmography, a non-invasive blood pressure sensor technology which records the first appearance of the pulse while deflating the occlusion cuff or the disappearance of pulses upon inflation of the occlusion cuff. Photoplethysmography utilizes an LED light source to record the pulse signal wave. The choice of this method was based on the available expertise, as well as its cost

effectiveness. It is also technically easier to accomplish compared to direct blood pressure measurement. Furthermore, tail cuff plethysmography allowed measurements to be made in young rats, which otherwise would have been technically difficult with cannulation, Several previous studies have demonstrated that blood pressure measured by tail-cuff plethysmography shows excellent correlation with blood pressure simultaneously obtained by intra-arterial direct measurement (Ikeda, Nara and Yamori 1991, Pfeffer, Pfeffer and Frohlich 1971).

The tail- cuff system used in our experiments measured blood pressure by determining the cuff pressure at which blood flow to the tail is eliminated. Tail-cuff blood pressure is defined as the cuff inflation pressure at which the waveform amplitude falls below a programmable percentage of its original amplitude for a specified number of waveform cycles. The blood pressure measurements were made with software purchased from the Medical Research Council (MRC) Blood Pressure Unit.

We undertook to measure the tail-cuff blood pressure of the subjects in a blinded fashion.

It is important to appreciate that measurement of blood pressure by photoplethysmography has a number of limitations. For example, it is relatively inaccurate because the readings are based solely on the amplitude of a single pulse and can only imprecisely measure the systolic blood pressure and the heart beat. Other limitations include over-saturation of the blood pressure signal by ambient

light, extreme sensitivity to the rodent's movement (motion artifact), and difficulty in obtaining adequate blood pressure signals in dark skinned rodents (pigmentation differentiation). Tail burns can result from close contact and prolonged exposure to the sensors. Furthermore, diastolic blood pressure cannot be measured by photoplethysmography since the technology records only the first appearance of the pulse (the systolic pressures). Occlusion cuff length, being inversely related to the accuracy of the blood pressure, is also a source of variability. Longer cuffs record lower than the actual blood pressure measurements. Hence, standard 20-mm long tail-cuffs were used throughout our experiments.

2.3.1

BLOOD PRESSURE MEASUREMENT IN ADULT OFFSPRING

Blood pressure was measured in cohorts of young and old animals. In adulthood blood pressure was assessed in 9 month old offspring. The animals were routinely handled every morning (08:30-10:30 am) and accustomed to the blood pressure measurement routine for one week before systolic blood pressure was measured using tail-cuff plethysmography (equipment purchased from Harvard Apparatus, Kent, UK). The animals were pre-warmed to an ambient temperature of 40ºC for approximately 5 minutes in a warming chamber.

As individual tail-cuff measurements were subject to variability because of the varied response of individual animals to the stresses (heating and restraint) involved in the procedure, we took a total of 4 blood pressure measurements per animal, and the arithmetic mean of 4 deflation cycles was used for the final computation of the systolic blood pressure, reducing variability.

The tails of the rats were passed through a cuff (20 mm in length and 10-mm in diameter) and immobilized by adhesive tape between a light source above and a photoresistor below the tail. Evaluated photoelectrically, blood flow in the tails produces oscillating waveforms that are digitally sampled 200 times per second per channel. The waveforms were displayed in real time on a monitor, and were computer-analyzed before and during a programmable routine of cuff inflation and

deflation. Programmable functions included (1) the number of waveforms analyzed to identify the amplitude and heart rate before each cuff inflation, (2) the number of preliminary unrecorded measurements, and (3) the number of recorded measurements per session.

In a further experiment, blood pressure was measured after animals had been pretreated with the MR antagonist spironolactone (Sigma-Aldrich, Poole, UK) 50mg/kg per day for seven days. Spironolactone was dissolved in mineral oil, and administered subcutaneously. Blood pressure measurements were then measured as described, in a blinded fashion.

2.4

HIGH SALT FEEDING AND BLOOD PRESSURE MEASUREMENT IN YOUNG OFFSPRING

In a separate cohort, blood pressure was measured in animals at 2 months of age. Measurements were done whilst animals were on standard laboratory chow (sodium 0.3%) and after 1 week of high salt diet (sodium 3%).

It is also important to note that the potassium contents of the standard chow and high salt rat chow was different. The standard rat chow contained 0.69% potassium, whereas the high salt diet had a potassium content of 0.51%.

The rationale for high salt feeding includes:

1. To better characterize the phenotype of the affected rats
2. To determine any changes resulting from the effects of a high-salt diet on the electrolytes (serum and urine) in affected subjects
3. 11β-HSD2 deficiency is associated with salt sensitivity

High salt diet was obtained from Special Diet Services (Essex, UK). The procedure used to measure blood pressure in young rats was otherwise similar to that describe above for old animals. The animals were sacrificed to collect tissue whilst on high salt diet. Hence, the long term effects after withdrawal of high salt feeding were not examined.

2.5

METABOLIC CAGE STUDIES

Metabolic cages allow monitoring mice and rats under standardized conditions, enabling accurate collection of samples (such as urine and feces), as well as estimation of food and water intake. In our experiments, a cohort of adult rats (aged 9 months old) was housed singly in standard metabolic cages (Harvard Apparatus, Kent, UK) to collect urine samples for electrolyte analyses.

2.5.1

EXOGENENOUS CORTISOL ADMINISTRATION

To investigate the effect of glucocorticoids on urinary electrolyte concentration, animals from the dexamethasone and control groups were singly housed in metabolic cages for 3 days for acclimatization. After 3 days, a single subcutaneous injection of cortisol (0.5 mg/kg) at 09.00 am. Urine was then collected for a further 24 hour period. The concentration of electrolytes in the urine was measured by the AVL 9180 electrolyte analyzer (Roche). Urinary aldosterone, renin and corticosterone using validated commercial ELISA kits (see section 2.8).

2.6

REAL-TIME POLYMERASE CHAIN REACTION

Animals were killed by decapitation in a fed state and kidneys were removed and immediately frozen in liquid nitrogen. All tissues were then stored in a -80ºC freezer for subsequent use. Whole kidneys were homogenized in TRIZOL (guanidinium thiocyanate, sodium acetate, phenol and chloroform) solution (Invitrogen, Paisley, UK) and total RNA extracted as per manufacturer's instruction (www.invitrogen.com). The basis of this method is that RNA is separated from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol and can be quantified.

RNA was quantified spectrophotometrically at OD260 using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. Nucleic acids are quantified using UV absorption using a spectrophotometer. In its simplest form the absorbance is measured at 260 and 280 nm. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. (An A260 reading of 1.0 is equivalent to about 40 µg/ml of RNA and the OD at 260 nm is used to determine the RNA concentration in a solution. RNA has its absorption

maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 2.1.)

Subsequently, we checked the RNA integrity by 1% agarose gel electrophoresis (0.5% Tris-Borate-EDTA buffer; 100 Volts/20 centimetres electrode distance; 45-60 minute runs). The RNA was visualised in the gel by addition of ethidium bromide. The RNA extracted were of excellent quality: intact RNA has sharp and clear 18S and 28S bands, with the 28S band approximately twice as intense as the 18S band under UV illumination.

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. In general, reverse transcriptase is a multifunctional enzyme with 3 distinct enzymatic activities: an RNA-dependent DNA polymerase, a hybrid-dependent exoribonuclease (RNase H), and a DNA-dependent DNA polymerase. In vivo, the combination of these 3 activities allows transcription of the single-stranded RNA genome into double-stranded DNA.

In our experiments, cDNA was thus synthesized from 1µg of RNA using the Quantitect Reverse Transcription kit (Qiagen), which incorporates a gDNA (genomic DNA) removal step prior to cDNA synthesis. The manufacturer's protocol was followed (www.qiagen.com). Briefly, the purified RNA sample is incubated in gDNA Wipeout Buffer and gDNAse at 42°C for 2 minutes to effectively remove contaminating genomic DNA. After genomic DNA elimination, the RNA sample is ready for reverse transcription using a master mix prepared from Quantiscript

Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C for 15 minutes and is then inactivated at 95°C for 3 minutes. The resultant cDNA was then used in quantitative, real-time PCR.

Real-time PCR quantification was carried out with the Lightcycler 480 real-time PCR system (Roche Applied Science) on 384 well plates. The Lightcycler performs the RT-PCR in small-volume glass capillary tubes, contained within a rotor-like carousel, that are heated and cooled in an airstream (Wittwer et al. 1997, Wittwer, Fillmore and Hillyard 1989). The carousel is rotated past a blue light-emitting diode, and fluorescence is read by three photodetection diodes with different wavelength filters that allow the use of spectrally distinct fluorescent probes (Wittwer et al. 1997, Wittwer et al. 1989).

We used TaqMan Gene Expression Assay primers and probes purchased from Applied Biosystems. TaqMan probes consist of a fluorophore covalently attached to the 5’-end of the oligonucleotide probe and a quencher at the 3’-end. The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler’s light source via FRET (Fluorescence Resonance Energy Transfer). Whilst the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals.

TaqMan probes anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to

the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in real-time PCR is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

The gene expression primer-probes used in the assays are shown in Table 2-1.

Table 2-1 List of primer-probes used in real-time PCR (Applied Biosystems).

Gene	Gene symbol	Assay ID
11-beta-hydroxysteroid dehydrogenase type 2	HSD11B2	Rn00492539_m1
11-beta-hydroxysteroid dehydrogenase type 1	HSD11B1	Rn00567167_m1
Glucocorticoid receptor	NR3C1	Rn00561369_m1
Mineralocorticoid receptor	NR3C2	Rn00565562_m1
Serine/threonine-protein kinase 1	SGK1	Rn00570285_m1
Epithelial sodium channel 1-alpha	SCNN1A	Rn00580652_m1
Epithelial sodium channel 1-beta	SCNN1B	Rn00561892_m1
Epithelial sodium channel 1-gamma	SCNN1G	Rn00566891_m1
Hepatocyte nuclear factor 4-alpha	HNF4A	Rn00573309_m1
erythropoietin	EPO	Rn01481376_m1
Hypoxia-inducible factor 1, alpha subunit	HIF1A	Rn00577560_m1
Hypoxia-inducible factor 2,	HIF2A	Rn00576515_m1

alpha subunit		
Hypoxia-inducible factor 1, beta subunit	HIF1B	Rn00562847_m1
GATA binding protein 2	GATA2	Rn00583735_m1

Primers for the hnf4a-P2 Assay were custom-designed and ordered from Applied Biosystems:

forward primer CTTGGTCATGGTCAGTGTGAAC;

backward primer AGGCTGTTGGATGAATTGAGGTT;

reporter sequence ACGTGTCATAAGGACTCGC-FAM.

Tata binding protein, tbp (Assay ID Rn01455648_m1) was used to normalize the mRNA levels of the genes of interest. (Preliminary experiments established that tbp mRNA expression did not alter with prenatal dexamethasone treatment when normalized to the control genes 18SrRNA and β-actin.) Serial 1:2 dilutions (with RNase-free water) of stock cDNA (pooled from all samples) were used to create a standard curve. No-template negative controls (nuclease-free water) and negative RT controls were added to the real-time plate. Each sample was run in duplicate and the mean value of the duplicates was used to calculate the transcript level.

The error for the standard curve thus obtained using the Lightcycler480 system was generally <0.01, and the standard crossing point cycle threshold (Cp) for the duplicate samples was 0.3 or less, which are well within the accepted ranges for validity for real-time PCR.

2.7

11β-HSD2 ENZYME ASSAY

11β-HSD2 is an exclusive 11β-dehydrogenase, and uses NAD as a cofactor to convert corticosterone (B) to 11-dehydrocoticosterone (A). Fresh kidneys were homogenized in a medium containing 20% glycerol, 4 mM tris (hydroxy- methy1) methylamine (Tris) and 1mM EDTA (pH 7.5). Protein content was then quantified using the Bradford protein assay, which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

To initiate the reaction, tissue homogenate (final reaction concentration of 0.2mg protein/ml) was incubated at 37^{o}C with 400μm NAD and 10 nM [^{3}H]-B in an assay buffer (10% glycerol, 300mM sodium chloride, 1mM EDTA and 50 mM Tris; pH 7.7). The reaction was terminated by placement on ice after 10 minutes. Steroids were extracted by ethyl acetate and quantified by high-performance liquid chromatography (HPLC) in mobile phase (65% water, 15% acetonitrile, 25% methanol). The ratio of A/ (A+B) steroids was used to calculate the enzyme activity

of renal 11β-HSD2, expressed as pmol product/mg protein/minute. All reagents used were of HPLC grade (Rathburn Chemicals, Walkerburn, Scotland).

2.8

ENZYME LINKED IMMUNOASSAYS AND ELECTROLYES

PLASMA ERYTHROPOIETIN CONCENTRATION

Samples were obtained between 8:30 and 10:00am from 9 month old animals in a fed state. Blood was collected by tail-tipping into heparinized tubes and spun at 5000 g for 5 minutes to obtain plasma. The samples were stored at -20ºC for subsequent analysis using a validated Rat Erythropoietin ELISA kit (USCN Life Science, Wuhan, China). The interassay and intraassay coefficients of variation were 7.3% and 6.6%, respectively. The sensitivity of the assay was less than 0.7 ng/ml.

Blood to determine plasma concentrations of renin, aldosterone, corticosterone and electrolyte concentrations was collected in the morning between 9:00 and 10:00 am. After tail tipping, free-flowing drops of blood were collected into heparinized Eppendorf tubes and spun immediately (2000g x 10 min). Electrolytes were measured using an AVL 9180 electrolyte analyzer (Roche, Switzerland) within 1 hour. Plasma samples to measure aldosterone and renin concentrations were stored at

-20ºC for subsequent analyses. Plasma renin was measured by enzyme linked immunoassay (ELISA) using a validated kit supplied by USCNLIFE (Wuhan, China), intraassay and interassay coefficients of variation <8%. Aldosterone was measured by a validated ELISA kit (Alpha Diagnostics International, Texas, USA). Intraassay and interassay coefficients of variation were <6% and <10%, respectively. Corticosterone levels were measured by a validated ELISA kit (AssayPro, St. Charles, Montana, USA). Intraassay and interassay coefficients were <5% and <8% respectively.

2.9

HISTOLOGY

Whole fresh kidneys were fixed in formalin for 24 hours and embedded in paraffin using standard procedures. Multiple adjacent 4 mm sections of the mid-transverse kidney were cut and mounted on glass slides. The sections were stained with hematoxylin and eosin. Glomeruli and tubule segments were identified based on positional and morphological criteria by a blinded observer. Morphometric measurements were taken using MCID image analysis using the Zeiss Axiovert 25 Phase Light Microscope. The range of magnification includes 5x, 10x, and 40x. The glomeruli number was counted under the 40x magnification field. The camera model employed was CoolSnap Photometrics with the software used being MCID Basic 7.0.

A total of 9 rats per group were sacrificed at 9 months of age. Transverse cuts through the mid kidneys, for consistency, were obtained for each rat and put on slides (3 slides per animal). The average number of glomeruli per 40x magnification field was counted in 5 random subcortical fields per slide in a blinded fashion.

(Zeiss). The average number of glomeruli per 40x magnification field per rat was then computed as the average of the 3 slides, and was used for comparison between the 2 groups.

In a similar fashion, the average diameter of all glomeruli under 40x magnification was measured in 5 random subcortical fields per slide. The average of 3 slides per animal was used for the subsequent comparison of the glomerular diameters between the dexamethasone-programmed group and their control litter-mates.

2.10

IMMUNOHISTOCHEMISTRY

Immunostaining was performed on 4mm mid-transverse sections of paraffin-embedded whole kidney. Sections were dewaxed in xylene and rehydrated through graded alcohols and subjected to pressure cooking in 0.01 M citrate (pH 6.0) buffer for antigen retrieval. Endogeneous peroxidase activity was inactivated in 3% (vol/vol) hydrogen peroxide in methanol for 30 minutes, followed by blocking of endogenous avidin-binding sites using an avidin/biotin kit (Vector Laboratories, Peterborough, UK), according to the manufacturer's protocol. Nonspecific staining was blocked by incubation with appropriate normal serum (Autogen Bioclear UK Ltd.) diluted 1:5 in TBS containing 5% BSA (Sigma-Aldrich Co. Ltd., Dorset, UK) for 30 min. Sections were incubated with polyclonal sheep anti- 11bHSD-2 antibody (dilution 1: 1000) overnight in a humidified chamber at 4°C. After two 5 min washes in TBS, the sections were incubated for 30 min with biotinylated anti-sheep antibody, diluted in normal serum. This was followed by two further 5 min washes in TBS, and incubation for 30 min with Streptavidin-horse-radish peroxidase at 1:1000 (Dako, Ely, UK) diluted in TBS. Staining was visualized using 3,3-diaminobenzidine tetrahydrochloride chromagen (DAB Substrate kit, Vector Laboratories,

Peterborough, UK). Sections were counterstained with haematoxylin, dehydrated in graded alcohols and immersed in xylene before mounting in Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). To ensure reproducibility of results and allow accurate comparison of immunostaining between groups, sections of kidney from control and dexamethasone-treated animals were processed in parallel. Appropriate negative controls were included, by replacing them with polyclonal sheep anti- 11βHSD-2 antibody with appropriate normal serum, to ensure that any staining observed was specific.

2.11

STATISTICS

All data are expressed as means ± SEM. Data were compared using unpaired student's t tests or one-way ANOVA followed by Newman-Keuls post-hoc multiple comparisons test, where appropriate. Values were considered significant at $p<0.05$. GraphPad software version 4.0 was used.

Determination of the smallest sample size was calculated using an online statistical resource (http://www.stat.ubc.ca). This calculation is based on the standard sample size calculation for unpaired sample means. (Fundamentals of Biostatistics, 6th edition, by Bernard Rosner), where n, the minimal sample size required is calculated as follows The letter "z" represents a standard normal distribution. Alpha represents the probability of a Type I error **(**taken to be 0.05). Beta represents the probability of a Type II error (taken to be 0.20). Sigma represents the standard deviation and, D is the clinically relevant difference (taken as 5 mmHg)**.** The values of sigma (the standard deviation) and mean blood pressure were based on previous estimations in studies of blood pressures of dexamethasone-treated offspring by measured by the tail-cuff method (O'Regan 2004).

$$n = \frac{\left(\sigma_1^2 + \sigma_2^2\right) \times \left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{D^2}$$

This calculation yielded a minimum sample size of 6 rats per group to satisfy the above statistical requirements for blood pressure measurements.

Similarly, the minimum sample size needed to detect a clinically relevant difference of 25% in gene expression was 6 rats per group based on the standard deviations derived from pilot real-time PCR studies (sigma less than 0.16 when means were normalized to control group).

CHAPTER 3

GLUCOCORTICOIDS AND OFFSPRING BLOOD PRESSURE

3.1

INTRODUCTION

The association between low birth weight and later life hypertension is strongly documented in the literature (Barker et al. 1993, Benediktsson et al. 1993, Seckl 1993, Seckl and Brown 1994, Seckl et al. 1995, Seckl 1997, Seckl et al. 1999, Seckl, Cleasby and Nyirenda 2000, Seckl 2001, Seckl and Meaney 2004, Seckl 2004, Seckl and Holmes 2007). Various animal models, using a variety of prenatal insults such as glucocorticoids (stress hormones), maternal hypoxia, maternal malnutrition and uterine artery ligation, all identify the prenatal period as a critical window for programming of offspring hypertension (Speirs, Seckl and Brown 2004).

Work previously done in our laboratory had used the daily injection of subcutaneous dexamethasone to pregnant Wistar rats as a model of fetal programming. The offspring were shown to have lower birthweight and developed later life hypertension and diabetes, two salient features of the metabolic syndrome which

contribute to increased atherosclerosis, risk of stroke and coronary artery disease (Bertram and Hanson 2001, Seckl and Holmes 2007, Bertram and Hanson 2002).

Using this model, key features of glucocorticoid fetal programming were defined. The last trimester of gestation (embryonic days 15 to 21 in the 3 week pregnancy of rodents) was identified as the critical period of programming hypertension and hyperglycemia (Seckl et al. 2000, Drake, Walker and Seckl 2005, O'Regan et al. 2004, Seckl 2004).

Glucocorticoid exposure in this period is sufficient and necessary for programming effects on offspring phenotype. Secondly, gender-specific programming effects on offspring have been documented. For instance, only prenatal dexamethasone-programmed female offspring had elevated hepatic angiotensin mRNA expression, plasma angiotensinogen and renin activity that developed with the hypertension (O'Regan et al. 2004). Thirdly, previous studies have provided insight into the pathophysiological mechanisms of hypertension in these offspring. Some important pathophysiological mechanisms implicated include reactive hypercortisolemia to stress (in part due to decreased feedback sensitivity in the HPA axis), greater sympathetic nervous response to stress, and greater increases in plasma renin and angiotensinogen (in female offspring) (O'Regan et al. 2004).

Other investigating groups, using different paradigms, have found decreased nephron numbers (Brenner, Garcia and Anderson 1988, Gilbert et al. 2005, Langley-Evans, Welham and Jackson 1999, Ortiz et al. 2003, Vehaskari, Aviles and Manning 2001,

Wintour et al. 2003, Woodall et al. 1996, Woods et al. 2001, Woods, Weeks and Rasch 2004) and mass, hyperaldosteronemia, impaired endothelium- dependent vasorelaxation (Schulz, Anter and Keaney 2004, Goodfellow et al. 1998, Leeson et al. 2001, Cripps, Martin-Gronert and Ozanne 2005, Panza et al. 1990) and decreased expression of renal 11β-HSD2 enzyme to be important in programming.

What is emerging from these studies, however, is that excessive exposure to glucocorticoids prenatally appears to effect coordinated changes in glucocorticoid action in the tissues. For instance, tissue-specific GR expression is affected; decreased GR expression in the hippocampus and the amygdala decreases the negative feedback of plasma hypercortisolism and leads to a higher basal rise in cortisol (Welberg et al. 2001). In the liver, GR expression was increased. This, coupled with the increased 11β-HSD1 enzyme in livers observed in marmoset primates, may contribute to increased liver gluconeogenesis, in part mediated by upregulation of Pck2 (Nyirenda et al. 1998), a rate-limiting enzyme in gluconeogenesis (Pilkis and Granner 1992) and an important glucocorticoid target.

The aims of the studies in this chapter were to confirm the effects of prenatal overexposure to glucocorticoids on offspring blood pressure and to examine the hormonal and electrolyte characteristics associated with this phenotype.

3.2

RESULTS

3.2.1

THE EFFECT OF PRENATAL DEXAMETHASONE ON BIRTH WEIGHT, MATERNAL WEIGHT GAIN AND LITTER CHARACTERISTICS

In accord with previous studies, offspring of dams that were treated with dexamethasone in the last trimester (days 15 to 21 gestation) had a modest but significant 10% reduction in their birth weight ($p<0.0001$) (see Fig 3-1).

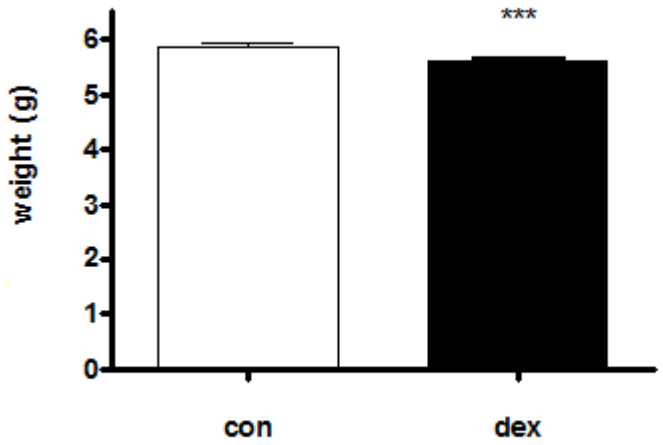


Figure 3-1 Effect of prenatal dexamethasone on 9 month old offspring body weight. ***=p<0.001 compared to controls. n=83 and n=97 for control and dexamethasone-treated group respectively.

Dams that received dexamethasone during pregnancy had significantly reduced weight gain once dexamethasone injections were started, compared to those that received vehicle (Table 3-1). This effect has been observed previously, and may be reflect a direct effect of dexamethasone on fetal growth, resulting in smaller fetuses with intrauterine growth retardation (Reinisch et al. 1978). Alternatively, the decrease in gestational weight gain may result from the effects on the mother, as dexamethasone has been shown to reduce maternal food intake (Jahng et al. 2008). Interestingly, although the maternal weight gained was 46% lower in the dexamethasone-treated dams (p=0.0006) 4 days after injections were begun, the maternal weight gained at 7 days (after injections were begun) was only 37% (p=0.0004) lower in the dexamethasone-treated group, suggesting some degree of 'catch-up' in maternal weight gain, possibly due to placental hypertrophy, fetal catch-up growth, or both.

Group	Maternal weight gained after 4 days of injections, grams	Maternal weight gained after 7 days of injections, grams
Control	48.83±4.67 (n=9)	98.83±6.11 (n=9)
DEX	26.29±1.95*** (n=9)	62.29±14.16*** (n=9)

Control (vehicle injections)

DEX (dexamethasone)

Table 3-1 Effect of prenatal dexamethasone on the maternal weight gained after injections were started on Day 15 of gestation. The weight gain of the pregnant dams was followed up after 4 days (Day 19 of gestation) and 7 days (Day 21 of gestation) respectively. ***=$p<0.0001$ when compared to controls.

Litter characteristics such as gestation length, litter size or male: female ratio in offspring were not affected by maternal treatment with dexamethasone at the dosage used (p=0.55, p=0.96 and p=0.99 respectively).

Group	Gestational Length, days	Litter Size (no. of pups)	Male: Female Ratio
Control (n=9)	22±0.15	13.50±1.03	1.65±0.35
DEX (n=9)	22±0.11	13.57±0.92	1.66±0.73

Table 3-2 Effect of prenatal dexamethasone on gestational length, litter size and male to female ratio of pups.

3.2.2

THE EFFECT OF PRENATAL DEXAMETHASONE ON POSTNATAL OFFSPRING WEIGHT AND KIDNEY WEIGHT

At birth, the offspring were culled to 8 males per litter. No further manipulations were made to the mothers and animals were kept under similar conditions. The offspring were weaned at the age of 21 days. By age 1 month, the dexamethasone - programmed offspring had caught up in weight with their control litter-mates ($p=0.70$). Henceforth, when examined at 5 months and 9 months old, the body weight in dexamethasone-programmed offspring was not significantly different from that in controls under standard dietary conditions ($p=0.75$ and $p=0.42$ respectively).

Group	Body Weight at 1 week, grams	Body weight at 1 mth, grams	Body weight at 5 mths, grams	Body weight at 9 mths, grams
Control (n=9)	26.80±0.65	47.60±0.75	584.6±15.45	669.0±29.3
DEX (n=9)	22.80±0.93**	46.90±1.07	576.6±17.98	642.6±13.3

Table 3-3 Effect of prenatal dexamethasone on body weight at 1 week, 1 month, 5 months and 9 months respectively. **= $p<0.01$ compared to the control group.

Prenatal treatment with dexamethasone was associated with a subtle (10%) but significant decrease in kidney weight at 1 week ($p=0.0005$). However, when normalized to body weight, there were no differences in kidney weights between dexamethasone-programmed offspring and controls, either at 1 week of age or in adulthood at 9 months.

A

Group	1 week body wt grams	1 week kidney weight, grams	1 week normalized kidney weight g/kg
Control (n=9)	26.80±0.65	0.335±0.009	12.52±0.32
DEX (n=9)	22.80±0.93**	0.280±0.005***	12.36±0.34

B

Group	9 mth body wt grams	9 mth kidney weight, grams	9 mth normalized kidney weight g/kg
Control (n=9)	669.4±29.3	4.19±0.17	6.30±0.18
DEX (n=9)	642.6±13.3	4.00±0.09	6.24±0.14

Table 3-4 Dexamethasone-treated offspring vs. control litter-mate kidney and body weights at 1 week (A) and 9 months (B) of age **=$p<0.001$; ***=$p<0.0001$ compared to controls.

3.2.3

THE EFFECT OF PRENATAL DEXAMETHASONE ON OFFSPRING BLOOD PRESSURE, URINARY SODIUM EXCRETION AND PLASMA ELECTROLYTES

Offspring systolic blood pressure was determined by tail-cuff plethysmography. In 9 month old adult offspring, there was a significant increase in the systolic blood pressure (12 mmHg) in the dexamethasone -programmed animals when compared to controls (p=0.008).

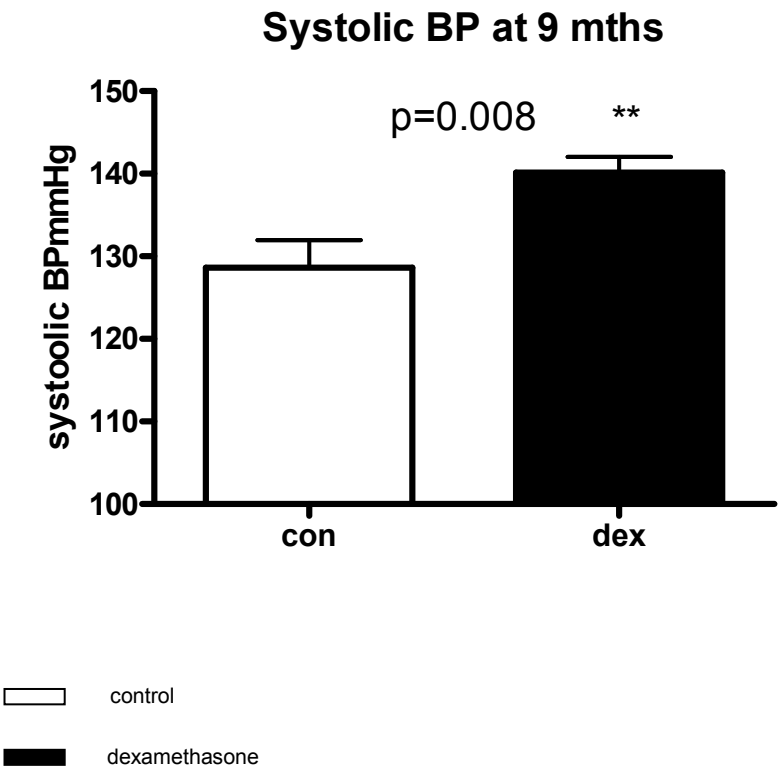


Fig 3-2 There was a significant increase in the systolic blood pressure in the dexamethasone -programmed animals when compared to controls ($p<0.05$). n=9 per group.

We examined urinary sodium excretion in 9 month old offspring using metabolic cages. Despite having higher blood pressures, the dexamethasone-programmed animals had a similar rate of urinary sodium excretion (dexamethasone-treated group, 1.22±0.09 mmol/24 hours; controls, 1.21±0.15 mmol/24 hours; p=0.93. This is consistent with either a shift of the pressure-natriuresis curve to the right or a gentler slope in the pressure-natriuresis curve, implying a lesser degree of renal sodium excretion (since the dexamethasone-programmed offspring have a higher blood pressure and would hence be expected to have a higher, not similar rate of urinary sodium excretion). Urine output (dexamethasone-treated group, 16.67±1.52 mls; controls, 16.67±1.52; p=1.00) and drinking rates (dexamethasone-treated group, 36.7±4.2 mls; controls, 30.0±3.6 mls; p=0.76) were not significantly different between the dexamethasone-programmed group and controls.

Plasma electrolytes were examined as an index of mineralocorticoid activity. Although there was no difference in plasma sodium ($p=0.76$), plasma potassium was 0.6 mmol/L lower in animals that were exposed to dexamethasone prenatally ($p=0.03$).

Group	Sodium (mmol/L)	Potassium (mmol/L)
Control	143.1±0.5 (n=10)	5.10±0.2 (n=10)
DEX	143.3±0.4 (n=10)	4.50±0.2* (n=10)

Table 3-5 Effect of prenatal dexamethasone on plasma concentrations of electrolytes in 9 months old offspring. *= $p<0.05$ when compared to controls.

Animals were placed on spironolactone at the age of 12 months. At this age, baseline blood pressure measurements were higher than those obtained at 9 months of age, but animals that were born to mothers that received dexamethasone during pregnancy continued to have higher blood systolic blood pressure compared control rats (DEX, 152.2±4.0; Control, 141.8±3.1 mmHg; p=0.06). However the difference in blood pressure between the groups was completely abolished after a week's treatment with spironolactone (DEX, 143.1±2.9; Control, 143.3±2.1 mmHg; p=0.89).

To examine whether these changes tracked from early life, we next examined a separate cohort of younger animals at age 2 months. In these younger animals, systolic blood pressure, plasma sodium and plasma potassium were not different between the dexamethasone -programmed offspring and the controls. (p=0.98, p=0.33 and p=0.51 respectively).

3.2.4

THE EFFECT OF PRENATAL DEXAMETHASONE ON SALT SENSITIVITY

In order to further characterize the effect of prenatal dexamethasone treatment on renal sodium handling and blood pressure control, we compared the effect of high-salt feeding on blood pressure in the 2 month old offspring that had been prenatally exposed to dexamethasone or vehicle.

Group	Blood pressure mmHg Standard Diet	Blood pressure mmHg High Salt Diet
Control (n=8)	118.8±3.1	120.8±2.5
DEX (n=8)	118.6±1.8	129.0±2.7 *

Table 3-6 Effect of prenatal dexamethasone on salt-sensitivity in 2 month old offspring on standard 0.3% sodium diet and after 1 week of 3% sodium (high-salt) diet. Means were compared using 1-way ANOVA and Newman-Keuls post-hoc testing. *= $p<0.05$ when DEX on high salt group is compared to all other groups.

After one week of high salt diet (3% Na content vs. 0.3% Na in standard rat chow), the dexamethasone-programmed offspring developed higher systolic blood pressures (dexamethasone-treated group, 129.0±2.7 mmHg; controls, 120.8±2.5 mmHg; $p<0.05$) compared to controls. As observed in the older (9 month old) offspring, this salt-sensitive increase in blood pressure was accompanied by significant lower plasma potassium in the dexamethasone-treated group when compared to controls (decrease of 0.7mmol/L). There were no differences in plasma sodium between the 2 groups after high salt feeding, although the sodium levels in both groups were higher after 1 week of high salt feeding when compared to baseline.

Group	plasma sodium, mmol/L Standard Diet	Plasma sodium, mmol/L High Salt Diet
Control (n=8)	140.4±0.3	143.4±0.60*
DEX (n=8)	139.7±0.6	143.0±0.84 **

Table 3-7 Effect of prenatal dexamethasone on 2 month old offspring plasma sodium on standard 0.3% sodium diet and after 1 week of 3% sodium (high-salt) diet. Means were compared using 1-way ANOVA and Newman-Keuls post-hoc testing. * = $p<0.05$ when Control on high salt group is compared to both Control and DEX groups on standard diet. **= $p<0.05$ when DEX on high salt group is compared to both Control and DEX groups on standard diet.

Group	Plasma potassium, mmol/L Standard Diet	plasma potassium, mmol/L High Salt Diet
Control (n=8)	4.32±0.13	3.98±0.12
DEX (n=8)	4.22±0.07	3.28±0.20 *, **

Table 3-8 Effect of prenatal dexamethasone on 2 month old offspring plasma potassium on standard 0.3% sodium diet and after 1 week of 3% sodium (high-salt) diet. Means were compared using 1-way ANOVA and Newman-Keuls post-hoc testing. *= $p<0.001$ when DEX on high salt group is compared to both Control and DEX groups on standard diet. ** = $p<0.01$ when DEX on high salt group is compared to control on high salt group.

3.2.5

THE EFFECT OF PRENATAL DEXAMETHASONE ON OFFSPRING RENIN, ALDOSTERONE AND CORTICOSTERONE

The hypertension and hypokalemia seen in the dexamethasone-programmed offspring was indicative of increased mineralocorticoid activity in the kidney. This was supported by reduced plasma renin concentrations (decrease of 45%, p=0.01) and volume expansion, features of increased mineralocorticoid activity. Intriguingly, offspring of dexamethasone -treated dams had significantly lower 24-hour urine aldosterone concentration that controls (decrease of 55%, p=0.006); there was also a tendency for lower plasma aldosterone levels but this did not reach statistical significance (p=0.34).

Group	Renin (ng/ml)	Aldosterone (ng/ml)	Urine Aldosterone (ng/kg body wt/24hr)
Control (n=8)	43.8±5.9	363.3±47.6	303.6±47.0
DEX (n=8)	23.6±4.8*	298.7±46.4	136.1±27.0**

Table 3-9. Effect of prenatal dexamethasone on plasma renin, plasma aldosterone and 24 hour urine aldosterone in 9 months old offspring. *=$p<0.05$ and **=$p<0.01$ vs. control respectively.

9 am plasma corticosterone (DEX, 39.23±4.41; control, 39.00±4.19 ng/ml; p=0.97) and 24-hour urinary corticosterone (DEX, 65.39±5.79; control, 41.08±10.62 ng/ml/24 hr/kg body weight; p=0.06) were not significantly different between dexamethasone- treated and control litter-mates, although the later tended towards statistical significance.

3.3

DISCUSSION

In this chapter, our experiments were aimed at further characterization of the phenotype of the dexamethasone -programmed offspring. Only male offspring were used in these experiments. This is because dexamethasone -programming has been shown to result in gender-specific changes adult physiology, and, secondly, blood pressure in female rats varies with the oestrous cycle, and coordinating blood pressure measurements during the oestrous cycle would have been technically demanding.

In accord with previous studies using tail-cuff measurements, blood pressure in adult offspring was elevated in animals born to dams that were treated with dexamethasone, both at 9 months and 12 months. The degree of systolic blood pressure elevation (12mmHg), if extrapolatable to humans and viewed from a whole population rather than an individual basis, would contribute significantly to mortality and morbidity.

In our experiments, we did not detect a higher blood pressure at 2 months of age. Others in our laboratory have examined blood pressure from 4 months of age, whereby blood pressure changes are already observed. Baum and colleagues, using a very similar model of programming, but using dexamethasone administered

intraperitoneally at 200mcg/kg/day (twice the dosage used in our studies), have found that the programmed offspring developed higher blood pressures at 2 months.

We offer 2 reasons: firstly, the dexamethasone programmed offspring might indeed have higher blood pressures, but the difference in blood pressures in this younger cohort was numerically small and the study was insufficiently powered to measure any difference. Secondly, the programming model used in our experiments may differ fundamentally. For instance the degree of changes in parameters such as normalized renal weights, glomerular diameters and glomerular numbers were not observed when compared to some of the other programming models (results presented in Chapter 4). Without these additional renal perturbations, the degree of blood pressure difference may not have been detected in the younger cohort.

Nonetheless, our data support the idea that blood pressure changes amplify with age; although we did not detect blood pressure changes in the younger (2 month old) rats, the dexamethasone -programmed offspring had significantly higher systolic blood pressures at 9 months of age.

Although the baseline urinary sodium excretion over 24 hours was not different in adult control and the dexamethasone programmed offspring at 9 months, this is consistent with either a shift of the pressure-natriuresis curve to the right or a gentler slope in the pressure-natriuresis curve, implying a deficit in renal sodium excretion (since the dexamethasone-programmed offspring have a higher blood pressure and

would hence be expected to have a higher, not similar rate of urinary sodium excretion).

We thus examined salt-sensitivity in the younger 2 month rats which did not display blood pressure differences. Salt-sensitivity is widely held to be an important independent factor of cardiovascular morbidity and mortality, independent of blood pressure (Morimoto et al 2007, Weinberger et al 2001). Moreover, some investigators have noted the increased risk of developing high blood pressure in salt-sensitive individuals. In low birth weight individuals, an increased salt-sensitivity has been observed. It was thus relevant to investigate salt-sensitivity in our younger cohort of programmed offspring, before the development of higher systolic blood pressures.

Intriguingly, despite the lack of differences in blood pressure in the younger animals, "stressing" this cohort with high-salt feeding brought out the hypertensive phenotype, which suggested the presence of an underlying abnormality in salt-handling. Unfortunately, the cohort of younger rats was not followed up subsequently to see if the effect of short-term high salt feeding on blood pressure was transient or persistent.

Many mechanisms of salt-sensitivity have been proposed and study. Here we discuss 2 potential mechanisms relevant to our programming model, namely a higher level of sympathetic nervous activity with greater pressor activity (Campese et al 1993) and decreased renal 11β-HSD2 activity (Bailey et al. 2011). O'Regan has previously

demonstrated that in this model of programming, the vasculature (mesenteric arteries) displayed greater vasoconstrictive response to noradrenaline and other vasoconstrictors (O'Regan et al 2008). These programmed offspring also showed a greater response in blood pressure to catecholamines. This is in accord with the observations of Campese and colleagues. In their study of 11 salt-sensitive patients and 14 salt-resistant patients, they found increased vasopressor reactivity to noradrenaline, and suggested that this may be an important mechanism for salt-sensitivity.

Interestingly, Bailey and colleagues have used the 11β-HSD2 haploinsufficiency mice and found that these animals develop salt-sensitive hypertension, This recapitulates the salt-sensitivity observed when licorice, an inhibitor of 11β-HSD2 is administered in human subjects (Ferrari P et al 2001) and serves as a very useful model for dissecting the mechanisms of salt-sensitivity in 11β-HSD2 insufficiency.

Working on this model, Bailey et al have noted that a high salt diet causes a progressive rise in plasma corticosterone, which likely is involved in salt-sensitivity, given that GR antagonism, but not MR antagonism, prevented the rise in salt-sensitive blood pressure. Corticosterone levels were not measured during high salt diet in our animals, but we noted a rise in the plasma urinary corticosterone levels in the adult offspring, in accord with previous observations of hypercorticosteronemia (Levitt NS et al 1996)

Another interesting observation was that dexamethasone-programming was associated with significant hypokalemia, either spontaneously in adulthood or provoked by high-salt feeding in the younger offspring. Hypokalemia, in the context of hypertension, is typically seen with increased renal mineralocorticoid activity, where sodium is retained by the distal renal tubules in exchange for potassium excretion.

In our rat model, the dexamethasone-programmed group did not display hypernatremia when compared to controls (when fed on standard diet). This phenomena (known as mineralocorticoid escape) is also a common feature of mineralocorticoid excess in humans; primary hyperaldosteronism and excess exogenous mineralocorticoid result in transient renal sodium retention which then returns to neutral sodium balance within a few days. This “escape” from mineralocorticoid-induced renal sodium retention appears to be mediated largely by extracellular fluid volume expansion (Knox et al. 1980, Hall et al. 1984), but the exact mechanisms are poorly understood.

In accord with this apparent increased renal mineralocorticoid activity (and thereby expansion of the intravascular volume), plasma renin concentration was lower in the dexamethasone -programmed animals. Paradoxically, the animals had significantly lower 24-hour urine aldosterone concentration. The constellation of low-renin hypertension, hypokalemia and lowered aldosterone levels is reminiscent of the syndrome of apparent mineralocorticoid excess (Mune et al. 1995, Kotelevtsev et al.

1999), either due existence of an atypical hormone or a result of intrinsic overactivity of the mineralocorticoid system within the kidney.

Patients with the syndrome of apparent mineralocorticoid excess have impaired conversion of cortisol to cortisone due to absence of the enzyme 11β-hydroxysteroid dehydrogenase (Ulick et al. 1979, Campino et al. 2010). In vivo, cortisol circulates at concentrations up to 1000-fold higher than aldosterone. However almost all MR activation in vivo is mediated by aldosterone. Surprisingly, in vitro, cortisol activated MR with a potency similar to that of aldosterone. This observation, together with the biochemical findings in syndrome of apparent mineralocorticoid excess , suggested that the specificity of MR for aldosterone in vivo is mediated indirectly, with 11β-HSD "protecting" MR from cortisol by metabolizing it to cortisone, which does not activate MR (Funder et al. 1988, Stewart et al. 1988).

In the syndrome of apparent mineralocorticoid excess, the absence of 11-βHSD allows cortisol to activate MR, resulting in hypertension mediated by increased ENaC activity. The 11β-HSD2 gene, which is expressed in the same cells of the nephron that express ENaC, shows homozygous loss-of-function mutations in syndrome of apparent mineralocorticoid excess patients (Mune et al. 1995).

Chronic ingestion of large quantities of natural licorice can inhibit 11β-HSD2 and also results in hypertension with suppressed renin and aldosterone. This is due to the action of a licorice metabolite, glycerrhetinic acid (Stewart et al. 1988), which is a

potent inhibitor of 11βHSD, producing a phenocopy of syndrome of apparent mineralocorticoid excess.

The clinical features of syndrome of apparent mineralocorticoid excess (hypertension, hypokalemia, low renin, and low aldosterone) are also similar to those of Liddle Syndrome, an autosomal dominant disorder with early onset hypertension associated with hypokalemic alkalosis, decreased plasma renin activity, and low plasma aldosterone levels. Liddle syndrome is caused by mutations in either the β or the γ subunit of ENaC that delete their cytoplasmic C termini (Shimkets et al. 1994, Hansson et al. 1995).

Other groups have used dexamethasone-programming models and found increased sodium reabsorption upstream to the aldosterone-sensitive distal nephron (Baum M, 2008). Indeed, the aldosterone-sensitive distal nephron is involved in just the excretion of 2-3% of the filtered sodium load in the kidney, whilst the more proximal tubules are responsible for the excretion of 97% of the filtered load. Nonetheless, monogenic mutations in humans, which involve sodium and virtually all secondary causes of hypertension including primary hyperaldosteronism, renal artery stenosis, phaeochromocytoma, involve net increase of sodium reabsorption at the level of the aldosterone-sensitive distal nephron.

Importantly, the phenotype observed (low renin, low aldosterone, hypokalemic hypertension and salt-sensitivity) in our dexamethasone-programmed rat, is compatible with a decrease in distal nephron 11β-HSD2 . We also note that MR

antagonism by spironolactone abolished the rise in blood pressure in the 12 month old programmed offspring. Considered together, the aldosterone-sensitive distal nephron, in particular 11β-HSD2 and/or MR, are plausibly altered by dexamethasone-programming.

Indeed, other groups using uterine artery ligation or the maternal protein restriction model have found changes in the aldosterone-sensitive distal nephron, including decreased 11β-HSD2 (Bertram CE et al 2003, Baserga et al 2010). Hence, we postulate that changes in the aldosterone-sensitive distal nephron may be operative in mediating the blood pressure changes observed in our model. These potential mechanisms of mineralocorticoid dysregulation will be a focus of the next chapter.

3.4

SUMMARY

Previous studies have shown glucocorticoids program hypertension. The mechanisms are unclear. A number of studies (and normal physiology) suggest that the kidney is important in pathogenesis of hypertension. Our data in this chapter is also supportive of this observation. In particular, we have demonstrated that dexamethasone programming is associated with reduced plasma potassium concentration and increased salt-sensitivity. The unique phenotype of low renin, low aldosterone hypokalemic hypertension suggests increased intrinsic (intrarenal) mineralocorticoid activity, typical of syndrome of apparent mineralocorticoid excess. This concept, particularly the effect of dexamethasone on renal 11β-HSD2 expression, is the subject of the next chapter.

CHAPTER 4

EFFECTS OF PRENATAL DEXAMETHASONE TREATMENT ON RENAL 11β-HSD2 AND MINERALOCORTICOID SIGNALING IN THE KIDNEY

4.1

INTRODUCTION

In the previous chapter, we established that prenatal dexamethasone treatment in the last trimester predisposed male offspring to hypertension, and that this is characterized by hypokalemia, low renin and low aldosterone concentrations. This is indicative of either existence of an atypical hormone with mineralocorticoid-receptor activity or intrinsic overactivity of the mineralocorticoid system within the kidney, resembling SAME (Mune et al. 1995, Kotelevtsev et al. 1999). Patients with this disorder (SAME) have impaired conversion of cortisol to cortisone due to absence of the enzyme 11β-hydroxysteroid dehydrogenase (Ulick et al. 1979). SAME can also be induced by chronic ingestion of large quantities of natural licorice due to the action of a licorice metabolite, glycerrhetinic acid (Stewart et al. 1988), which is a potent inhibitor of 11β-HSD2.

The clinical features of SAME (hypertension, hypokalemia, low renin, and low aldosterone) are also similar to those of Liddle Syndrome, an autosomal dominant

disorder with early onset hypertension associated with hypokalemic alkalosis, decreased plasma renin activity, and low plasma aldosterone levels. Liddle syndrome is caused by mutations in either the β or the γ subunit of ENaC that delete their cytoplasmic C termini (Shimkets et al. 1994, Hansson et al. 1995)

The phenotype expressed by the dexamethasone programmed offspring (low-renin low aldosterone type hypertension, associated with hypokalemia) might therefore result from one of the following three possibilities:

1.) There is reduced renal 11β-HSD2 enzyme activity (similar to the syndrome of apparent mineralocorticoid excess).
2.) There is an increase in circulating glycerrhetinic acid-like factors (GALFs) leading to inhibition of renal 11β-HSD2 enzyme activity.
3.) There is an increase in cortisol or a similar mineralocorticoid-active steroid or factor in the dexamethasone programmed offspring.

We contend that either the first or second possibility is more likely because, as explored later in this chapter, this phenotype was associated with increased sensitivity to exogenous glucocorticoids, indicating that the renal 11β-HSD2 enzyme barrier is attenuated. The present chapter therefore explores the effect of prenatal dexamethasone exposure on expression of 11β-HSD2 and other mediators of mineralocorticoid signaling in the kidney.

4.2

RESULTS

4.2.1

THE EFFECT OF PRENATAL DEXAMETHASONE TREATMENT ON RENAL 11β-HSD1 and 11β-HSD2

Renal mRNA expression of 11β-HSD1 and 11β-HSD2 was compared in 1 week and 9 months old offspring of dams that received dexamethasone or vehicle during the last week of pregnancy. Antenatal dexamethasone was associated with a marked reduction in renal expression of 11β-HSD2 mRNA. This decrease was seen at one week of age, and persisted into adulthood (decrease of 36%, p=0.04 and decrease of 45%, p=0.04 in 1 week old and 9 months old offspring respectively). The decrease in 11β-HSD2 mRNA was accompanied by a parallel reduction (37%) in 11β-HSD2 enzyme activity (DEX, 4.41±0.36 protein; Control 2.76± 0.21 pmol product/ min/ mg; p=0.0015).

In contrast, prenatal exposure to dexamethasone had no significant effect on renal 11β-HSD1 mRNA expression compared to controls (p=0.3 and p=0.2 at 1 week and 9 months respectively). The absence of significant effect of prenatal dexamethasone on renal expression of 11β-HSD1 was in contrast to liver 11β-HSD1, which had been

found to be upregulated after prenatal dexamethasone administration, at least in marmosets (Nyirenda et al. 2009)

It is not known if prenatal dexamethasone has a direct or indirect effect in 'programming' the renal expression of 11β-HSD2. However, we note that the expression of 11β-HSD2 in the liver and adrenals was not significantly altered by prenatal dexamethasone administration ($p= 0.88$ and $p= 0.89$ respectively). This suggests that the 'programming' of this down-regulation of 11β-HSD2 expression observed in the kidney involves tissue-specific factors that influence the long-term expression 11β-HSD2 during a period of 'epigenetic plasticity' in early life.

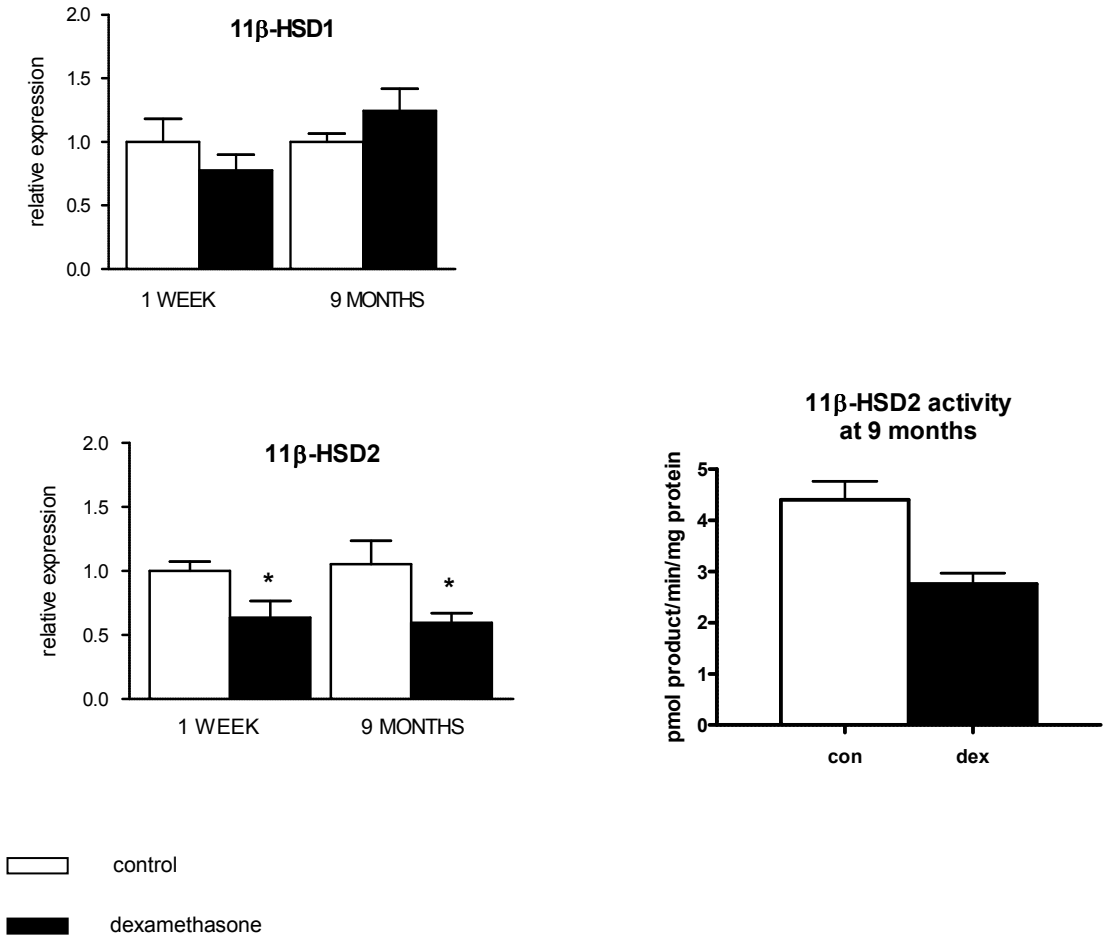


Fig 4-1 Effect of prenatal dexamethasone on renal mRNA expression of 11β-HSD1, 11β-HSD2 and renal 11β-HSD2 enzyme activity.

Renal 11β-HSD2 activity was measured in 9 months old offspring of dams that received dexamethasone (DEX) or control during the last week of pregnancy. Results represent mean±SEM; n=8 per group. *$p<0.05$ vs. control

4.2.2

THE EFFECT OF EXOGENOUS CORTISOL ON RENAL ELECTROLYTE HANDLING

Decreased 11β-HSD2 activity in the kidney would increase glucocorticoid access to MR and thereby promote sodium reabsorption and potassium excretion. To test whether the alteration in 11β-HSD2 activity described above was functionally important, we examined urine sodium and potassium concentrations in dex- treated and control animals after exogenous administration of cortisol. Cortisol will be easily metabolized in presence of adequate 11β-HSD2 but not if 11β-HSD2 activity is critically reduced.

In our metabolic cage experiments, baseline urine electrolytes (sodium and electrolytes) were measured over a 24 hour period. After the administration of a subcutaneous injection of cortisol (0.5 mg/kg), the urine electrolytes were measured for a further 24 hour period.

Rats prenatally exposed to prenatal dexamethasone showed a significantly greater fall in the ratio of sodium to potassium in the urine after cortisol administration, consistent with increased mineralocorticoid action from an attenuated 11β-HSD 2 enzyme pre-receptor barrier (Table 4-1).

In accord with increased sodium (and water) reabsorption with increased mineralocorticoid activity, we observed that cortisol administration was associated with a significant decrease in urinary sodium concentration ($p=0.006$). This increased sodium reabsorption (and hence decreased urinary sodium concentration observed) would suggest that this degree of 11β-HSD2 enzyme activity reduction (of 36%) allows exogenous cortisol at the dose used (0.5mg/kg) to increase mineralocorticoid activity leading to increased renal sodium and water reabsorption, a key mechanism in the development of chronic higher blood pressures in physiological models.

Although we expected significant kalliuresis (and increased urinary potassium excretion) after the administration of cortisol in the dexamethasone-treated animals, we did not observe this to be so (Table 4-3). This could likely be due to that the sodium content in the diet used (0.3% sodium chloride) was insufficient to allow marked kalliuresis to be expressed.

It is also noteworthy that the baseline urine potassium between controls and dexamethasone-treated animals were similar, despite the reported decreased 11β-HSD2 enzyme activity and higher mineralocorticoid activity which would result in kalliuresis. This is likely due to the lower plasma potassium levels in the dexamethasone-treated animals compared to the controls (DEX, 4.5 mmol/L; controls 5.1 mmol/L), leading to the similar baseline urinary potassium excretion observed.

In our metabolic cage experiments, there were no significant differences in 24-hour urine volume between the dexamethasone-treated group and controls at baseline, and after the administration of cortisol. We also did not detect any differences in bodyweight in the dexamethasone-treated and controls, both at baseline and at the end of the experiment.

Group	Baseline sodium/potassium ratio	Sodium/potassium ratio after cortisol
Control	0.30±0.03 (n=9)	0.25±0.03 (n=9)
DEX	0.31±0.03 (n=9)	0.21±0.02* (n=9)

Table 4-1 Effect of exogenous cortisol on urinary sodium/potassium ratio in 9 months old offspring.*p=0.01

Group	Baseline urinary sodium concentration mmol/L	Urine sodium concentration after cortisol mmol/L
Control	72.3±8.9 (n=9)	62.1±9.7 (n=9)
DEX	73.3±5.6 (n=9)	44.7±7.1** (n=9)

Table 4-2 Effect of exogenous cortisol on urinary sodium concentration in 9 months old offspring. **= p≤0.001

Group	Baseline urinary potassium concentration mmol/L	Urine potassium concentration after cortisol mmol/L
Control	239.6±23.1 (n=9)	209.1±24.6 (n=9)
DEX	243.9±16.7 (n=9)	218.9±16.5 (n=9)

Table 4-3 Effect of exogenous cortisol on urinary potassium concentration in 9 months old offspring.

4.2.3

THE EFFECT OF DEXAMETHASONE ON RENAL EXPRESSION OF THE MINERALOCORTICOID RECEPTOR AND GLUCOCORTICOID RECEPTOR

Previous studies have implicated MR and GR as important programming targets in various organs. In rodents, prenatal dexamethasone was associated with an increased expression of GR in the liver, possibly crucial in the upregulation Pck2, (a rate-limiting gluconeogenic enzyme) observed in the periportal region of the liver. However, we did not detect any increase (or decrease) in GR mRNA expression in the 1 week old or 9 months old rat kidneys. Similarly, we did not detect any significant changes in the expression of MR mRNA.

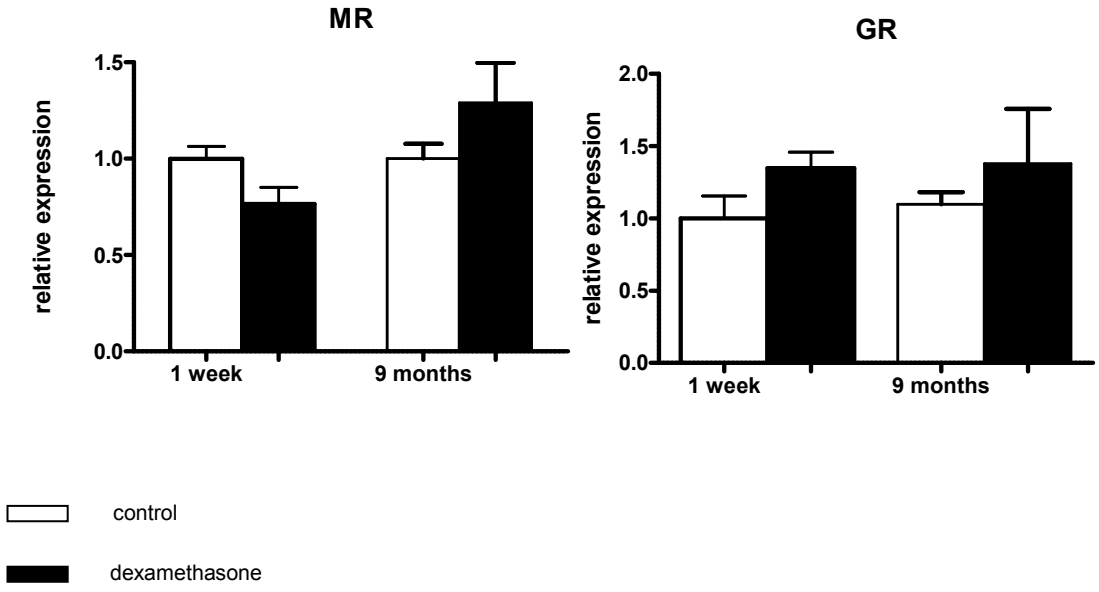


Fig 4-2 Effect of prenatal dexamethasone on the renal mRNA expression of GR and MR.

4.2.4

THE EFFECT OF DEXAMETHASONE ON RENAL EXPRESSION OF THE EPITHELIAL SODIUM CHANNEL (ENAC) SERUM- AND GLUCOCORTICOID-REGULATED KINASE (SGK1)

Sgk1 is an enzyme downstream of activated MR in the renin-aldosterone pathway which mediates the action of aldosterone in the distal renal tubular cell, leading to increased sodium reabsorption via ENaC. We hypothesized that Sgk1 mRNA expression in our rat model would be altered (upregulated) by the increased mineralocorticoid activation from reduced 11β-HSD2 expression in the kidney. However, renal Sgk1 expression was similar between rats that were exposed to dexamethasone prenatally and control animals (p=.42 and 0.27 at 1 week and 9 months respectively). The explanation for this is unclear, but it is possible that an enzyme other than Sgk1 may be operative in mediating the actions of activated MR. Indeed, in knockout mice lacking Sgk1, the predicted phenotype of salt-losing hypotension was not observed, indicating the existence of other downstream mediators of activated MR (Wulff et al. 2002).

Similarly, mRNAexpression of the various subunits of ENaC was not found to be statistically different between the prenatal dexamethasone treatment group and their control litter-mates. Although the levels of relative expression for all three subunits of ENaC (especially ENACα) tended towards an upward trend in 1 week DEX-treated animals (p=0.05), none of these changes quite reached statistical significance.

Moreover, the tendency for ENACα to be higher in the dexamethasone-treated animals did not persist in adult (9 months) offspring.

Nuclear MR activation leads to the subsequent transcription of various genes, including ENACs, which are crucial for transepithelial sodium transport. We note that ENaCs activity however, is not critically regulated at the mRNA level: it is the post-transcriptional modifications and transport and insertion of ENaC subunits into the luminal cell membrane that ultimately increases sodium (water) retention, leading to hypertension

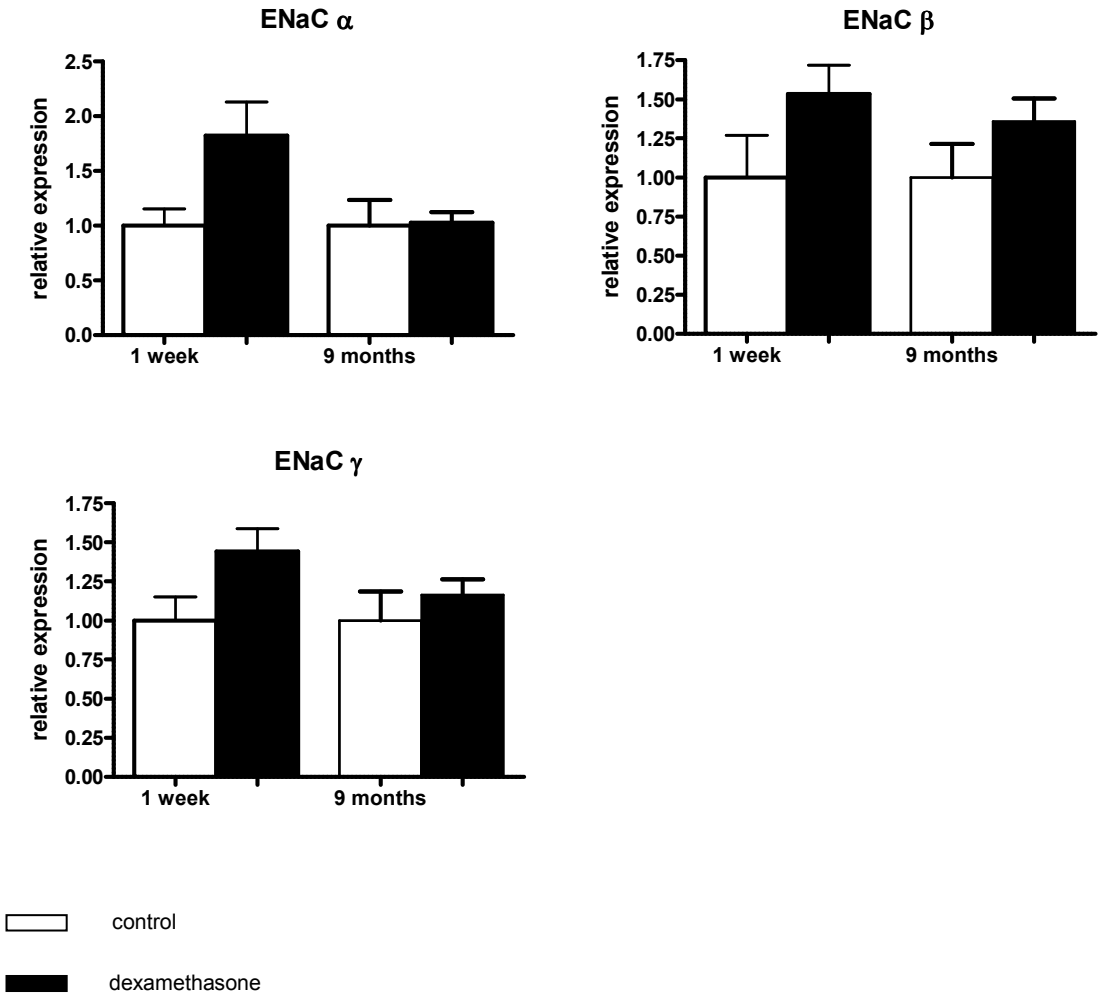


Fig 4-3 Effect of prenatal dexamethasone on the renal mRNA expression of ENaC (α, β, γ).

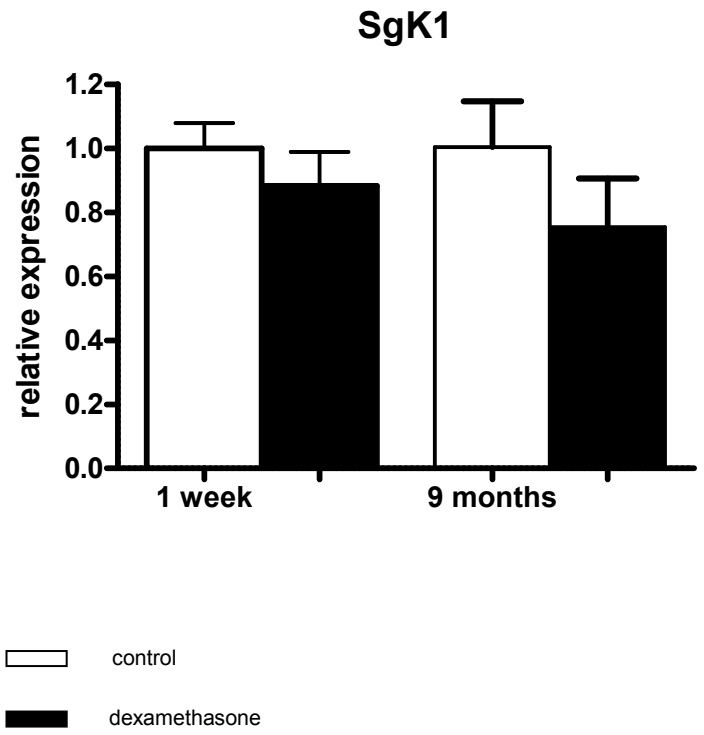


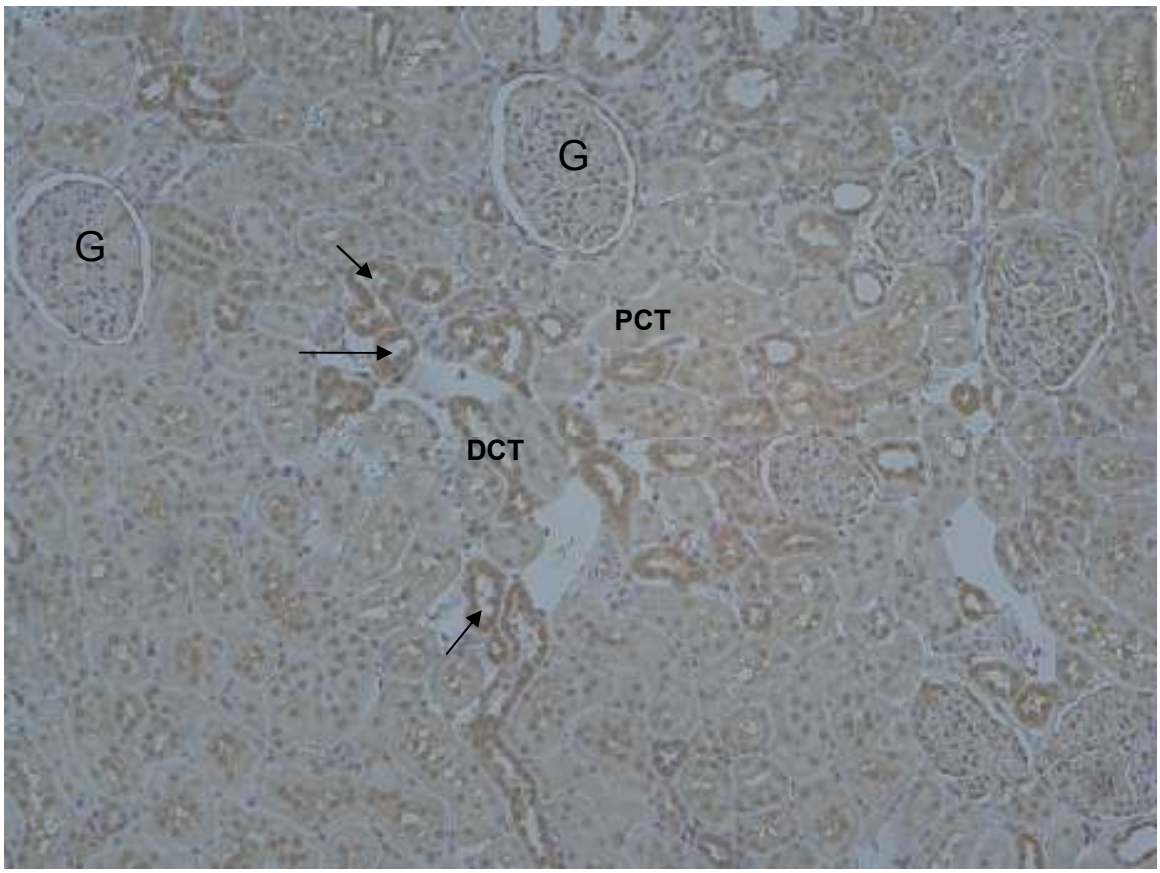
Fig 4-4 Effect of prenatal dexamethasone on the renal mRNA expression of Sgk1

4.2.5

IMMUNOHISTOCHEMISTRY AND HISTOLOGY

We employed immunohistochemistry to localize 11β-HSD2 expression within the kidney. Previous immunohistochemical studies in human kidney showed strong 11β-HSD2 immunoreactivity in the cortical distal convoluted tubules and collecting ducts. Strong staining was also observed in medullary tubules, which had the appearance of collecting ducts and the thick ascending limb of Henle's loop. This is consistent with the findings by other studies (Krozowski et al. 1995). Thus 11β-HSD2 co-localizes with the mineralocorticoid receptor in the distal nephron where it allows aldosterone to occupy its physiological receptor.

Our results were consistent with these findings: 11β-HSD2 was selectively expressed in the DCT (site of mineralocorticoid action), with no appreciable amounts of 11β-HSD2 detected ectopically in the PCT or glomerulus.



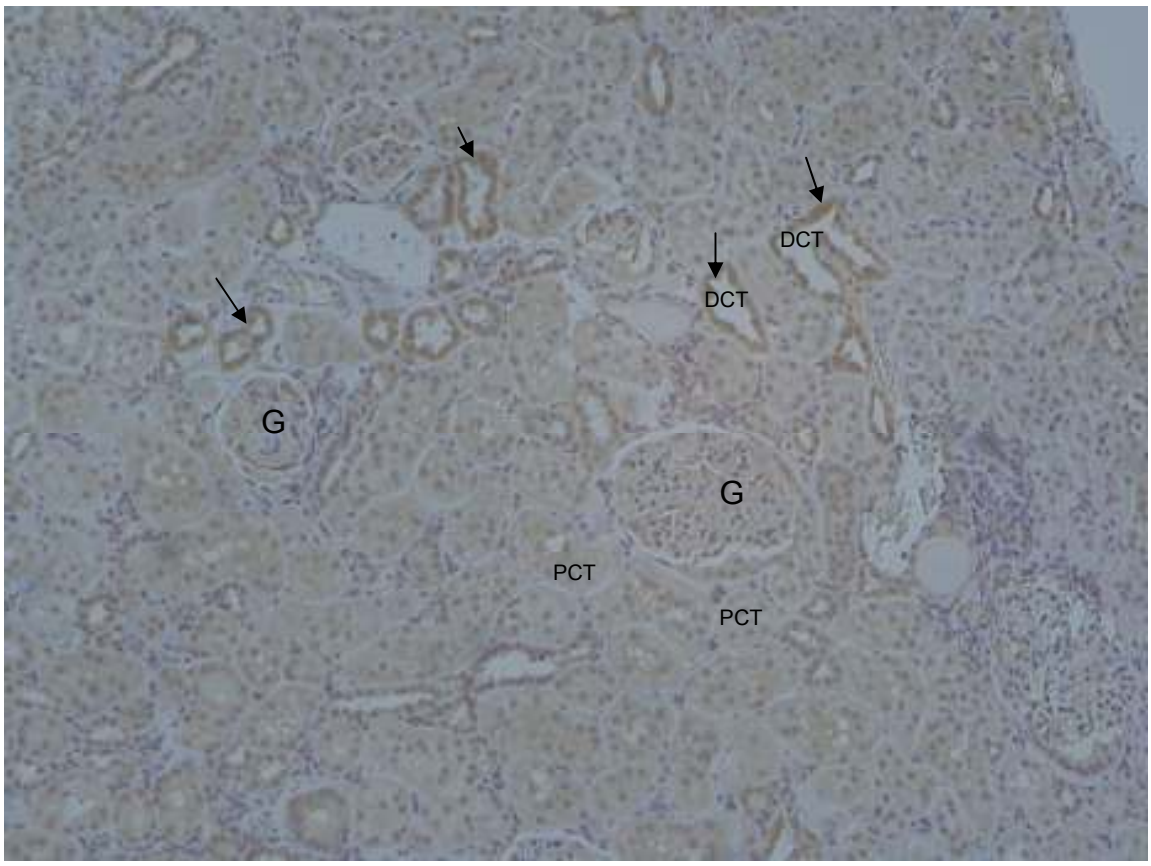
→ Arrow indicates areas of immunostaining

G Glomerulus
PCT Proximal tubule
DCT Distal tubule

Fig 4-5 Effect of prenatal dexamethasone on 11β-HSD2 distribution

In dexamethasone-treated rats, immunohistochemistry showed strong 11β-HSD2 immunoreactivity in the cortical distal convoluted tubules and collecting ducts. In addition, we did not detect differences in glomerular size (DEX, 10 190± 723 μm²; control, 10 370± 752 μm²; p=0.86) or numbers (DEX, 8.9± 2.1; control 9.4± 1.3 glomeruli per field under 25x magnification; p= 0.82) in dexamethasone-treated adult offspring at 9 months of age as compared to their control litter-mates (n=9 per group

We speculate that this may, at least in our model, be due to the fact that the normalized kidney weight (kidney weights normalized to bodyweight) was not different between the 2 groups. Since total body circulating blood volume is dependent on body weight, the total body circulating blood volume is proportionally reduced in the dexamethasone-treated animals, leading to normal, and not hyperfiltration of the glomeruli. This is consistent with the lack of glomerular hypertrophy and equal glomeruli numbers observed between the 2 groups at 9 months. At 9 months, the dexamethasone-treated animals had caught up in terms of body weight and absolute kidney weight (see Table 3-4 in Chapter 3), and there were no differences in kidney and body weights between the 2 groups.



→ indicates
areas of immunostaining

G Glomerulus
PCT Proximal tubule
DCT Distal tubule

Fig 4-6 11β-HSD2 distribution in control kidneys

4.3

DISCUSSION

Our study demonstrates that hypertension programmed by prenatal overexposure to glucocorticoids is associated with a permanent decrease in renal 11β-HSD2 mRNA expression and activity, without any effect on expression of GR, MR and 11β-HSD1 mRNAs. The reduction in renal 11β-HSD2 occurs in association with increased circulating glucocorticoids, as we have previously shown that DEX programmed rats have high plasma corticosterone concentrations [Levitt NS et al 1996, Nyirenda et al 1998].

11β-HSD2 plays a critical role in the kidney, protecting MR from activation by glucocorticoids, and attenuated 11β-HSD2 activity might therefore allow illicit stimulation of MR by endogenous cortisol (or corticosterone in rodents), leading to plasma volume expansion due excessive sodium retention. Indeed, hypertension in DEX-exposed rats was accompanied by hypokalemia and suppression of renin and aldosterone production.

Moreover, exogenous glucocorticoid administration elicited an acute mineralocorticoid response only in programmed rats, whilst treating the animals with spironolactone, an MR antagonist, eliminated the difference in blood pressure between the DEX group and control animals. In this respect, this model recapitulates SAME which traditionally occurs in patients with inactivating mutations in the 11β-HSD2 gene or after ingestion of liquorice, a naturally occurring inhibitor of 11β-HSD2 enzyme (Mune et al 1995, Stewart 1988).

In our rat model, prenatal DEX caused approximately 45% reduction in renal 11β-HSD2 mRNA and activity. This reduction is quantitatively similar to that seen in humans with

heterozygous mutations that induce salient features of the SAME (Li et al 1997)]. Moreover, polymorphisms in the 11β-HSD2 gene have been associated with essential hypertension in some populations, although this has not been consistently reproduced (Williams et al 2005, Brand et al 1998, Watson 1996).

The syndrome of apparent mineralocorticoid excess is inherited in an autosomal recessive fashion, with patients with this syndrome presenting early in childhood or adolescence (Lifton, Gharavi and Geller 2001, Stewart et al. 1996). However, there are also reports that parents of these patients (heterozygotes) can display features of SAME (low-renin low-aldosterone hypertension with hypokalemia), suggesting a gene dosage effect.

Similarly, inhibition of 11β-HSD2 activity with liquorice results in dose-dependent changes in blood pressure (Funder et al. 1988, Edwards et al. 1988, Stewart et al. 1987), (White, Mune and Agarwal 1997, Monder et al. 1989). It is therefore likely that the modest of reduction of 11β-HSD2 activity seen in dexamethasone-programmed rats contributes, at least in part, to the hypertension observed.

Our data showing that features of SAME can be provoked by high salt feeding in young programmed rats suggest that heterozygous individuals with11β-HSD2 mutation (who have subclinical deficiency of the enzyme) might have susceptibility to develop hypertension when exposed to physiological stressors (such as high sat diet). Further studies will be needed to explore this in humans.

Since the decrease in renal 11β-HSD2 mRNA expression in DEX-exposed rats was observed as early as 1 week of age, it is likely that this change was primary rather than secondary to other mechanisms, such as changes in blood pressure, especially important as the decrease in 11β-HSD2 in may be a compensatory response to the hypotension seen in this model when measured by radiotelemetry in "stress-free" environments.

Indeed, at a young age (2 months), there was no significant difference in blood pressure between young DEX-exposed and control rats under standard dietary conditions (although we do not rule out the possibility that important small differences in blood pressure may remain undetected). Nevertheless, offspring of dams that received DEX displayed exquisite sensitivity to high salt feeding, which brought out the hypertensive phenotype and provoked hypokalemia, suggesting that the decrease in renal 11β-HSD2 activity was functionally significant from an early age.

Taken together, our data indicate that prenatal DEX programs reduced 11β-HSD2 and increased salt sensitivity from birth. Children with IUGR have high plasma cortisol-to-cortisone ratio, indicative of reduced 11β-HSD2 activity (Houang et al 1989) . Since the kidney is the major site of 11β-HSD2 production postnatally (Whitworth et al 1989), it is tempting to speculate that reduced renal 11β-HSD2 expression might explain, at least in part, the association between low birth weight and later susceptibility to hypertension.

As in humans, although the deficit in 11β-HSD2 expression was seen early, the increase in blood pressure in our DEX programmed rats only become apparent upon increasing dietary salt content, and with aging, presumably as a result of the additive effects of subsequent environmental factors. This is consistent with recent proposals that aging amplifies the effect of low birth weight on blood pressure (Huxley et al 2000, Davies 2006).

Other studies have suggested reduction in nephron number as a mechanism of prenatal programming of hypertension (Brenner, Garcia and Anderson 1988). Optimal quantification of nephron size would include the length and size of the distal nephron. In this respect, our studies did not achieve detailed estimates of renal mass and nephron size/number. Based on our measurements, we were not able to detect lower glomerular numbers or glomerular hypertrophy in the dexamethasone-treated adults. This lack of changes seen in the glomerular size and number departs from the observations in other models, where structural kidney changes are observed.

One factor may be because the programming of structural changes in the kidney changes is dependent on the dose of glucocorticoids used. Baum et al have used intraperitoneal dexamethasone at a dose of 200 mcg/kg /day dexamethasone in their programming model (double that used in our model) and found a modest 20% decrease in the glomerular number using unbiased stereomicroscopic methods. In their model, with these additional changes in glomerular numbers, hypertension was seen in their 2 months old animals, departing somewhat from our observations. Further experiments in glomerular apoptosis, collagen deposition and albuminuria should help confirm whether other important changes in renal structure and physiology relevant in fetal programming models are present in our model.

Previous studies have indicated that glucocorticoid (or stress) during pregnancy programs coordinated changes in whole organism physiology, perhaps reflecting fetal adaptations to increase chances of surviving the projected extrauterine environment. These adaptations may, at least in part, be mediated by tissue specific

changes in glucocorticoid action. For example, dexamethasone-programming in the rat alters GR, MR and 11β-HSD21 in various tissues (Welberg and Seckl 2001, Levitt et al. 1996, Seckl 2004, Edwards et al. 1996, Seckl et al. 1995). Similarly, prenatal glucocorticoid excess in marmosets induced permanent increases in liver GR and/or 11β-HSD1 expression in key metabolic tissues, which might contribute to the pathogenesis of hyperglycaemia (Nyirenda et al 2009).

Interesting, in the rat kidney, only 11β-HSD2 expression was altered by prenatal dexamethasone treatment, with other targets (such as GR, MR or 11β-HSD1) unchanged. Our study therefore identifies 11β-HSD2 as the key programming target gene in hypertension. The mechanisms by which dexamethasone induces permanent changes in 11β-HSD2 expression are unclear, but these may involve either a direct effect on the 11β-HSD2 gene itself, or secondarily by regulating key transcriptional factor(s).

One potential direct effect might involve epigenetic modification. The 11β-HSD2 promoter has CpG islands (Alikhani-Koopaei et al. 2004), and increased methylation of the CpG islands have been shown to result in decreased expression of 11β-HSD2 mRNA. Prenatal environmental insults (such as maternal undernutrition) during critical periods of development have been shown to affect methylation status of genes (including GR), and it is therefore plausible to speculate that fetal exposure to glucocorticoid excess might have a similar epigenetic effect. Future experiments and knowledge of 11β-HSD2 regulation (e.g., promoter methylation) will help with unraveling the exact mechanism involved.

Downstream signalling components of the mineralocorticoid signalling components in the kidney include SGK-1 and ENAC. Patients with Liddle's syndrome, a monogenic disease in which ENAC are not degraded via ubiquination due to a mutation involving the recognition site on ENAC by Nedd4-2, develop low-renin, low-aldosterone, hypokalemia and hypertension, features similar to those observed in our programming model. It was thus important to measure the expression of ENAC subunits and SGK-1, as it remained a possibility that dexamethasone programming per se might upregulate ENAC and SGK-1, apart from the usual upstream activation by MR. However no statistically significant upregulation of ENAC and SGK-1 gene expression was found experimentally in our programmed animals at age 1 week or 9 months.

Ideally, it would be important to investigate apical ENAC abundance and activity as well as SGK phosphorylation states in our programming model to confirm that downstream targets of MR are indeed upregulated; experiments to document increased water reabsorption in the distal tubules using in vivo microperfusion techniques would also strongly support our hypothesis that water reabsorption in the aldosterone-sensitive distal tubule is important in our programming model.

We note, however, that the regulation of ENAC by 11β-HSD2, is not straightforward (Bailey et al. 2008) . In the 11β- HSD2 null mice, although sodium retention with increased ENAC activity was observed initially, by 80 days old, these mice were volume contracted, not expanded as expected, and the increased ENAC activity was abolished. Instead there appeared to be a switch in the mechanism of hypertension

from increased sodium and water retention to increased vasoconstriction with adrenergic blockade relieving the hypertension in the older mice.

Other limitations also affect the interpretation of our data. In our experiments, only male offspring were investigated and gender-differences in the programming of hypertension have been observed broadly across many species; female offspring thus may have different mechanisms of hypertension programming. Additionally, it is unknown whether the high salt diet itself affects 11β-HSD2 mRNA expression. It is also important to establish if microalbuminuria, a strong predictor of cardiovascular risk, is increased in our programming model.

Finally, aside from the kidney, 11β-HSD2 is also expressed in a number of organs, notably the heart and vasculature where it may play an important role to regulate blood pressure. It is therefore important to investigate whether prenatal dexamethasone affects expression of 11β-HSD2 mRNA and activity in these tissues, in particular cardiovascular structures such as the blood vessels and the myocardium, and whether this contributes to the hypertensive phenotype.

Despite the limitations mentioned, our data indicate that renal 11β-HSD2 is an important candidate gene in fetal programming of hypertension and provides a novel mechanism for fetal origins of adult salt-sensitive hypertension. If extrapolated to humans, these data suggest that programmed hypertension may be amenable to measures to reduce features of mild SAME such as blockade of MR or its downstream targets and that such offspring may be specifically sensitive to sodium-induced hypertension.

4.4

SUMMARY

In this chapter, we showed that prenatal dexamethasone exposure reduces renal expression 11β-HSD2. This decrease in 11β-HSD2 expression was present in young (1 week old) offspring, and persisted till adulthood. Not surprisingly, 11β-HSD2 enzyme activity, as measured in an in vitro assay was significantly reduced. This modest degree in renal 11β-HSD2 activity reduction appears to be physiologically relevant: the administration of exogenous cortisol, acting as a mineralocorticoid, induced a significant decrease in the sodium: potassium ratio in the 24-hour urine collection.

The decrease in 11β-HSD2 activity likely plays an important pathogenic role in the higher blood pressures observed in dexamethasone-programmed offspring. Similar findings by other investigators employing different models of programming highlight the important general role that 11β-HSD2 (and the mineralocorticoid signalling pathway) may play in mediating hypertension in fetal programming (Bertram et al 2003, Baserga et al 2010).

CHAPTER 5

EXCESSIVE PRENATAL GLUCOCORTICOID EXPOSURE PROGRAMMES OFFSPRING ERYTHROPOEITIN AND INCREASES RED BLOOD CELL MASS

5.1

INTRODUCTION

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Poiseuille's Law states the relationship between blood pressure and viscosity: the higher the viscosity, the higher the blood pressure.This raises the interesting possibility that the hypertension observed in the dexamethasone- programmed offspring could be in part contributed by yet another renal mechanism - increased erythropoietin production with increases in red cell mass, leading to subsequent hyperviscosity. In the previous chapters, we investigated the effects of prenatal dexamethasone on blood pressure, mediated through alteration in renal tubular 11β-HSD2 expression. In this chapter, we hypothesized that prenatal glucocorticoid exposure might programme renal erythropoietin (EPO) production and red cell mass was examined

Erythropoietin is the key hormone that stimulates hematopoietic stem cells in the bone marrow, liver and spleen, to increase erythropoeisis. During development, erythropoietin synthesis takes place in fetal liver and kidney, but postnatally the kidney is the main organ for production of erythropoietin (Beru et al. 1986).

The regulation of erythropoietin in the kidney has been investigated and summarized previously (Goldberg, Dunning and Bunn 1988). Tissue hypoxia critically induces erythropoietin production in chronic anemia. At the molecular level, erythropoietin production is determined by gene transcriptional activity. The erythropoietin gene has a 3' enhancer that contains a hypoxic- responsive element (HRE). The hypoxia inducible factors (HIFs), which include HIFα and HIFβ subunits, play an important regulatory role in response to tissue hypoxia. HIFs interact with other transcriptional factors such as HNF4α, GATA and Sp1 at the enhancer site, allowing for the hypoxia-driven induction of erythropoietin gene expression (Ebert and Bunn 1999).

Hepatocyte nuclear factor 4 alpha (HNF4α) is constitutively expressed in the sites of erythropoietin production (the adult kidney and the fetal liver). HNF4α binds to the erythropoietin (EPO) 3' enhancer site which lies next to the HIF-binding site (Blanchard et al. 1992) . HIF and HNF4α interact together with transcription factors like P300, form a stable multifactorial complex at the 3' enhancer and reinforces the promoter-enhanced contact, leading to the greater transcriptional activity and expression of erythropoietin (Ebert and Bunn 1998).

Recently, prenatal dexamethasone exposure was found to significantly elevate hepatic HNF4α mRNA expression in both fetal and adult offspring (Nyirenda et al. 2006). This increase resulted from increased expression of isoforms derived from the P1 HNF4α promoter. In contrast, isoforms derived from the P2 promoter were markedly suppressed by dexamethasone. This increase in HNF4α mRNA expression was postulated to mediate the effects of prenatal dexamethasone in programming hyperglycemia.

Given the possibility that prenatal dexamethasone may also, via similar molecular mechanisms, up regulate HNF4α expression in the kidney, we also investigated the effects of prenatal dexamethasone on renal HNF4α.

The possibility that renal erythropoietin production may be programmed by prenatal dexamethasone is an exciting one. Beyond the possible effect on blood pressure, increased erythropoietin has been independently shown to increased cardiovascular risk, and thus provides a novel mechanism to explain the higher cardiovascular and stroke mortalities observed in adults who were born of low birth weight. .

5.2

RESULTS

5.2.1

EFFECT OF PRENATAL DEXAMETHASONE ON RENAL AND HEPATIC ERYTHROPOEITIN EXPRESSION

EPO mRNA expression was measured in the offspring in the neonatal period at the age of 1 week, and in the adult offspring at 9 months of age. At 1 week, offspring of dams that received dexamethasone in the last week of gestation had significantly higher EPO mRNA expression compared to those that were born to control mothers (increase of 2.4 fold; $p=0.01$). This trend of higher EPO mRNA expression was also observed in the 9 month old cohort. Again, the dexamethasone-treated offspring had higher EPO mRNA expression compared to their control offspring (increase of 1.9 fold; $p=0.03$).

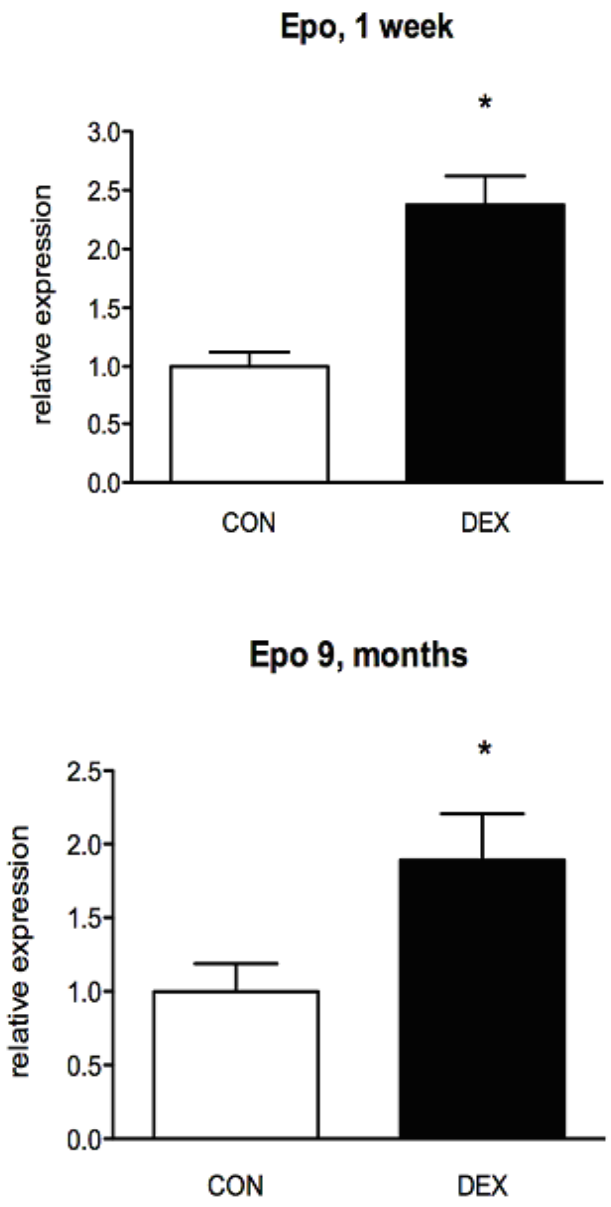


Fig 5-1 Effect of prenatal dexamethasone on EPO mRNA expression in 1 week old and 9 months old offspring. *=$p<0.05$. n=6 per group.

To confirm that the increase in renal EPO mRNA expression was functionally important we measured the concentration of EPO in plasma in 9 month old animals. Indeed, prenatal exposure to dexamethasone was associated with a significant increase in circulating plasma EPO levels (increase of 1.6 fold; p=0.04). As EPO is thought to be regulated primarily at the level of gene transcription (Wiesener et al. 1998), the increase in plasma EPO concentration is consistent with the increase expression of EPO mRNA observed.

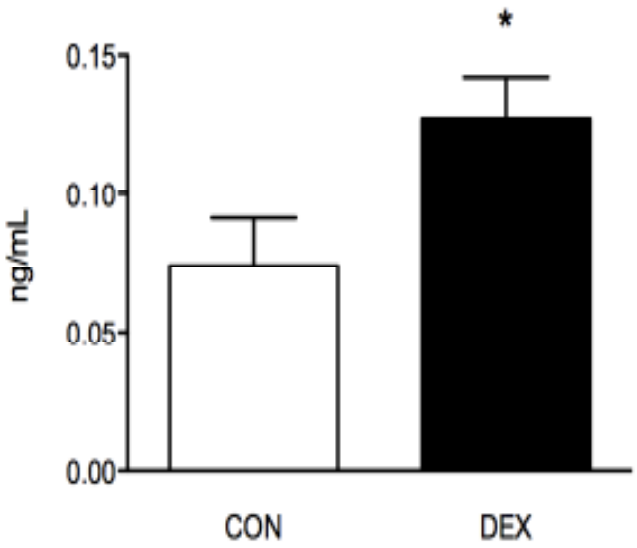


Fig 5-2 Effect of prenatal dexamethasone on plasma EPO concentration in 9 month old offspring. *=p<0.05. n=9 per group.

The fetal liver is the other key site of EPO production, although postnatally only the kidney is the main source of EPO. In order to get better understanding of the effect of glucocorticoids on EPO expression, we studied the effect of dexamethasone on hepatic expression of EPO in the liver, at embryonic day 21 (E21) and in adulthood. EPO was readily detected in fetal liver, and, as in the kidney, dexamethasone treatment caused a significant increase EPO mRNA expression at E21 (increase of 2.2 fold; p=0.03).

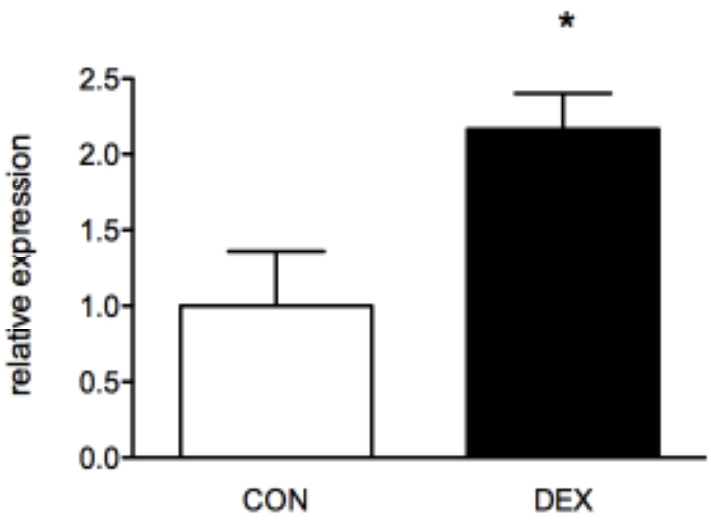


Fig 5-3 Effect of prenatal dexamethasone on hepatic EPO mRNA expression at E21. n=6 per group.

In contrast, we did not detect appreciable amounts of EPO mRNA in the adult livers of either control or dexamethasone-treated group. This is consistent with previous studies that have shown that the adult liver does not produce EPO.

5.2.2

THE EFFECT OF PRENATAL DEXAMETHASONE ON RENAL HNF4α ISOFORM EXPRESSION

Previous work in the laboratory had shown that prenatal dexamethasone treatment permanently increases expression of HNF4α in the liver. Specifically, dexamethasone increased expression of the isoforms derived from the P1 promoter, while those driven by the P2 promoter were significantly suppressed. In order to determine whether prenatal dexamethasone exposure had similar effects on expression of HNF4α in the kidney, we measured expression of these isoforms in kidneys of rats that were born to mothers that received dexamethasone or vehicle during the last week of pregnancy. Offspring of dams that received dexamethasone during gestation had significantly higher renal HNF4α mRNA expression than offspring of control dams (increase of 51%, p=0.03). Importantly, the increase in HNF4α mRNA was observed in one week old offspring (increase of 64%, p=0.01). This suggests that transient prenatal dexamethasone treatment of maternal dams in the third week of gestation upregulates the expression of renal HNF4α mRNA expression in the young (1 week old) offspring, and this effect (upregulation renal HNF4α mRNA expression) persists on into adulthood. This is similar to what has been previously observed in the liver.

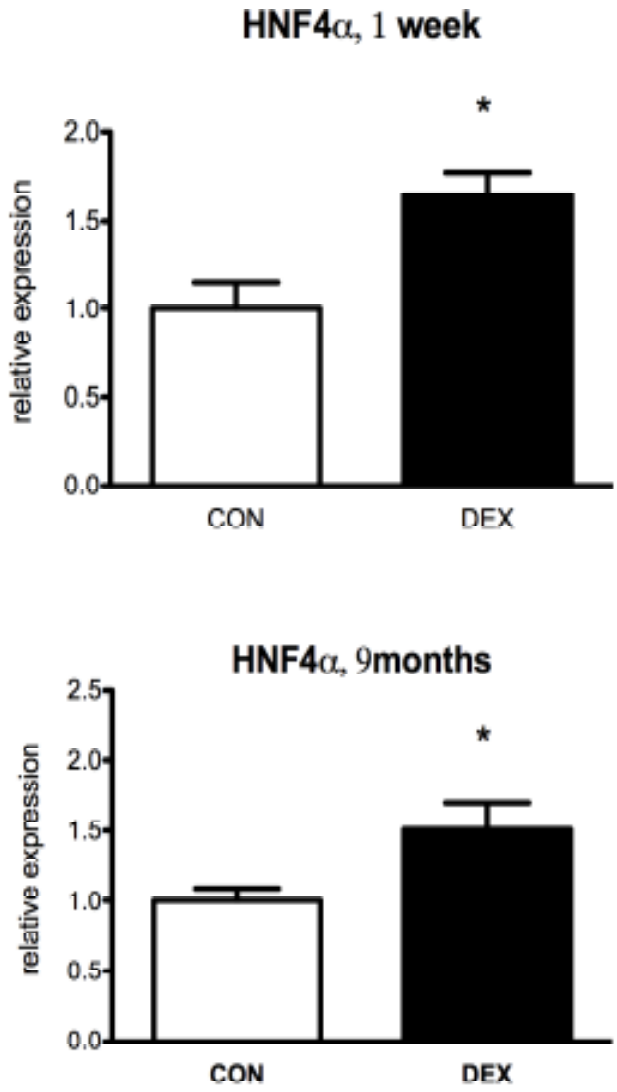


Fig 5-4 Effect of prenatal dexamethasone on renal HNF4α mRNA expression. *=$p<0.05$. n=6 per group.

However, when we probed for specific isoforms, we observed that all the HNF4α isoforms in the kidney appeared to derive from the P1 promoter, with no appreciable P2 transcripts detected, both in control or dexamethasone -exposed rats. This

observation was consistently seen at embryonic day 21 (E21), 1week and 9 month old offspring.

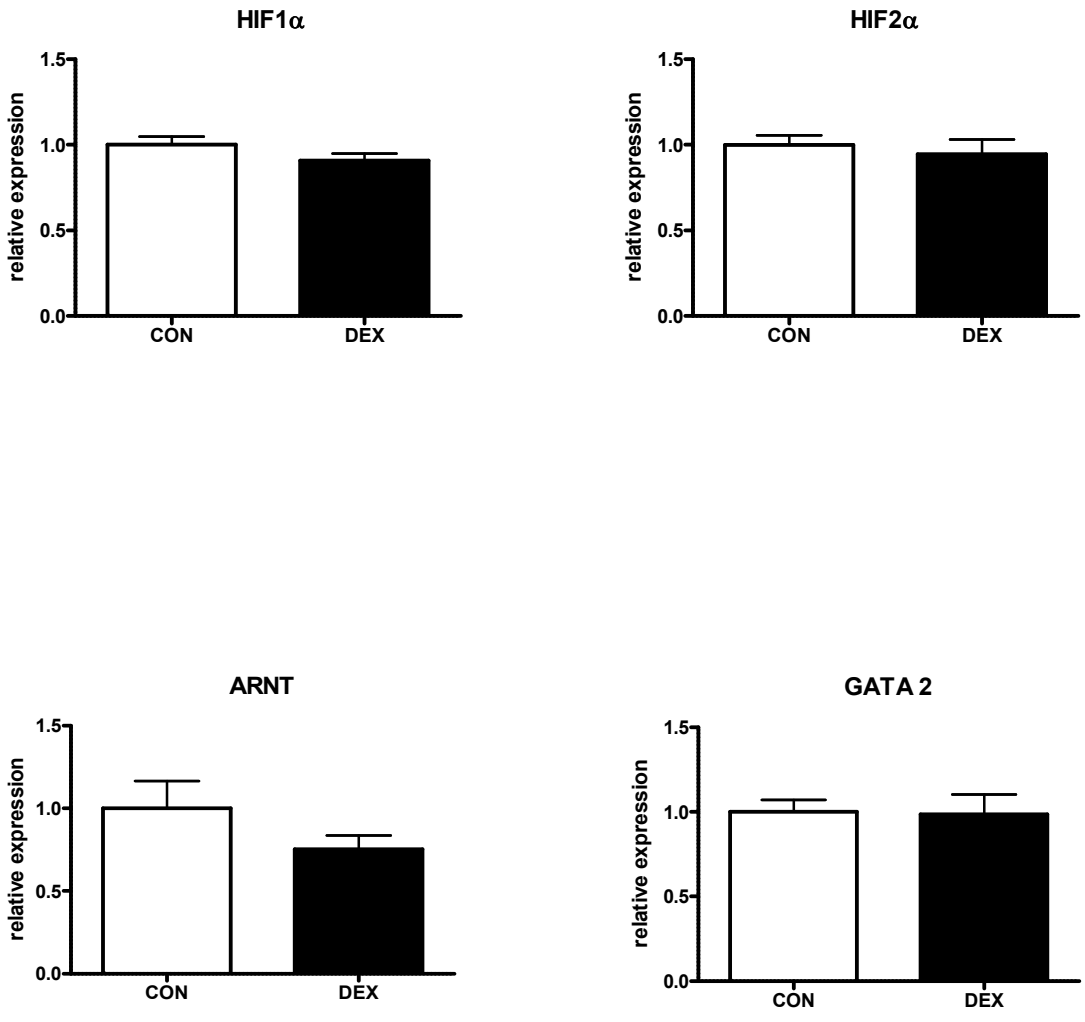
5.2.3

EFFECT OF PRENATAL DEXAMETHASONE ON TRANSCRIPTIONAL FACTORS INVOLVED IN EPO REGULATION

We also examined the effect of prenatal dexamethasone treatment on renal expression of other transcription factors that are involved in the regulation of EPO synthesis from the kidney. These include hypoxia-inducible factor 1, alpha subunit (HIF1α), hypoxia-inducible factor 2, alpha subunit (HIF2α) aryl hydrocarbon receptor nuclear translocator (ARNT) and GATA binding protein 2 (GATA2). These transcriptional factors, especially HIFs, form a transcriptional complex with HNF4α at the EPO 3'enhancer and act synergistically to recruit other transcriptional coactivators.

There were no significant differences between offspring of dexamethasone -treated dams and control animals in renal expression of HIF1α, HIF2α, ARNT or GATA2 mRNA, both in the 1 week old offspring and the 9 month old offspring.

A


HIF1α
relative expression
1.5
1.0
0.5
0.0
CON
DEX
HIF2α
relative expression
1.5
1.0
0.5
0.0
CON
DEX
ARNT
relative expression
1.5
1.0
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CON
DEX
GATA 2
relative expression
1.5
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B

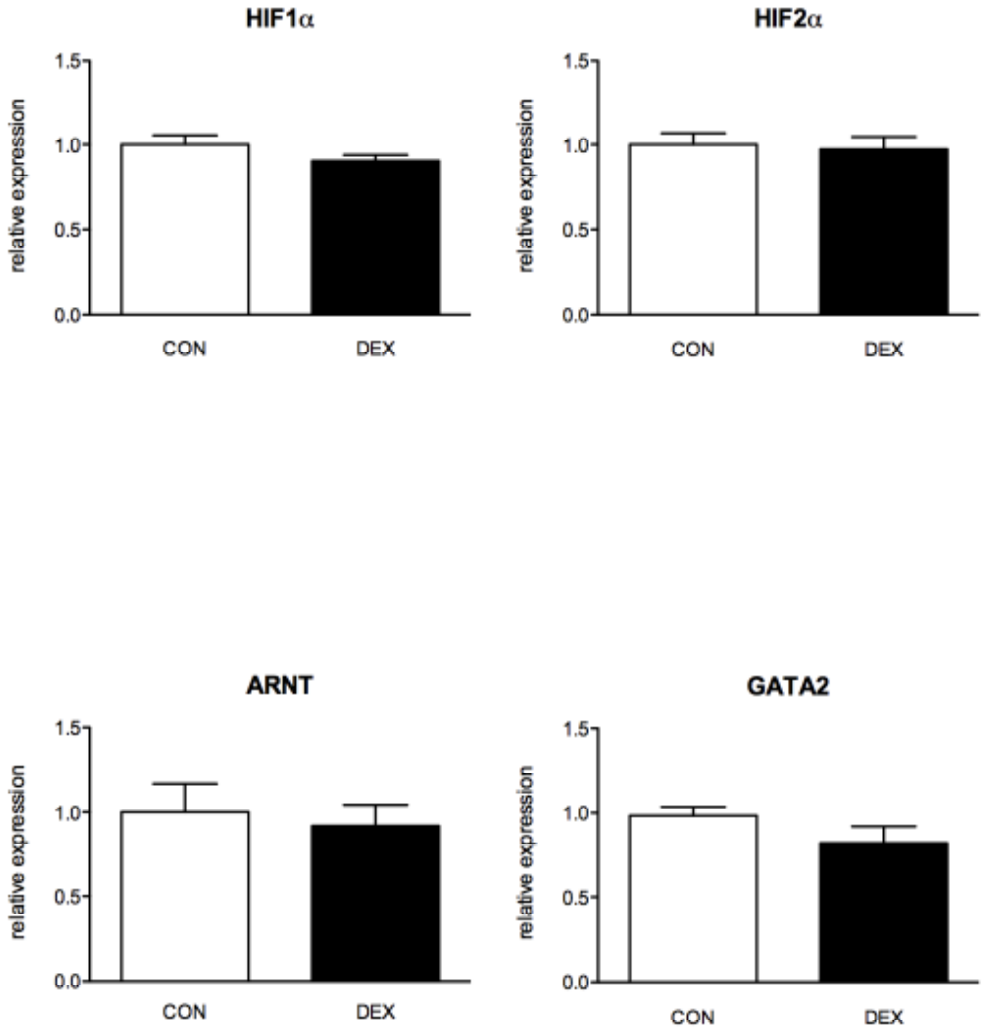

Fig 5-5 The effect of prenatal dexamethasone on transcriptional regulators of EPO. n=6 per group at 1 week (Figure A) and 9 months (Figure B).

Thus, of all the transcriptional regulators of EPO transcription, only renal HNF4α (P1-driven transcript) was upregulation by prenatal dexamethasone. This upregulation of HNF4α appears to be important in the increased EPO expression observed.

5.2.4

EFFECT OF PRENATAL DEXAMETHASONE ON BLOOD COUNT IN ADULT OFFSPRING

Since EPO is the main regulator of erythropoiesis, we next examined whether the increase in EPO synthesis following prenatal exposure to dexamethasone was associated with changes in red cell mass. The adult offspring of dams that were treated with dexamethasone during the last week of pregnancy had a significant increase in the number of circulating red blood cells (increase of 7%; p=0.02), hematocrit (increase of 8% p=0.004) and hemoglobin concentration (increase of 7%; p=0.02). This was accompanied by an increased reticulocyte count (increase of 10%; p=0.04). There were no differences in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) or mean corpuscular hemoglobin concentration (MCHC) between offspring of dexamethasone -treated dams and controls.

Prenatal dexamethasone treatment also resulted in the white blood cell (WBC) and platelet counts tending towards an increased trend despite not achieving statistical significance.

	Control	**DEX**	P value
WBC (10^9/L)	8.7±0.8	10.6±0.9	0.11
RBC (10^{12}/L)	8.8±0.2	9.4±0.1*	0.02
RET (10^9/L)	235.7±5.6	258.2±8.8*	0.04
HCT (%)	46.7±0.7	50.0±0.5**	0.004
HGB (g/dL)	16.2±0.2	17.3±0.2*	0.02
MCV (fL)	52.2±0.3	52.9±0.5	0.13
MCH (pg)	18.0±0.1	18.2±0.2	0.58
MCHC (g/dL)	34.57±0.13	34.35±0.14	0.35
PLT (10^9/L)	476.3±82.6	620.7±56.0	0.17

Table 5-1 Blood cell indices. *=$p<0.05$; **=$p<0.01$. n=9 animals per group.

HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet; RBC, red blood cell; RET, reticulocyte; WBC, white blood cell.

5.3

DISCUSSION

In this chapter, we investigated the effect of prenatal dexamethasone on erythropoeisis. Prenatal exposure to dexamethasone was also associated with increased expression of EPO mRNA in the kidney and fetal liver- the key sites of erythropoietin production. The increase of EPO mRNA was accompanied by a similar increase in plasma EPO concentrations in adult offspring. Although we hypothesized that the increase in systolic blood pressure could in part be mediated by increased plasma viscosity (by Poiseuille's Law), ultimately, we find that the absolute increase in hematocrit (by a meager 6%), although statistically significant, is unlikely to contribute as a factor to the increase in blood pressure observed through changes in viscosity. Nonetheless, a raised plasma erythropoietin per se has been associated with an increase in cardiovascular risk and mortality. Hence, it will be of clinical interest to investigate if similar changes in plasma erythropoietin are observed in low birth weight humans.

Interestingly, although prenatal dexamethasone permanently elevates hepatic expression of P1 HNF4α transcripts (Nyirenda et al. 2006) in the adult livers, (a finding which we replicated in our experiments), EPO is not detected in the liver postnatally, and over expression of HNF4α mRNA per se was not sufficient to maintain hepatic EPO production in the adult rats.

EPO is not stored preformed, and therefore any change in plasma EPO concentration results primarily from a change in transcription of the gene (Ebert and Bunn 1999). Although acute regulation of EPO transcription, mediated via HIF, has been extensively studied (Semenza 2001), the mechanisms that control steady-state production of EPO necessary to maintain the physiological daily renewal of red blood cells are less clear (Fandrey 2004). Similarly, determinants of developmental and tissue-specific expression of EPO are largely unknown.

Most studies suggest that HIF requires cooperation with other transcription factors to regulate of EPO expression. In particular, HNF4α, which is constitutively expressed in fetal liver and adult kidney, the major sites of EPO production, contributes importantly to the physiologic regulation of the EPO gene.

HNF4α binds adjacent to HIF binding-site in the mouse EPO 3' enhancer where it functions synergistically with HIF1 through direct protein-protein interaction and by recruitment of transcriptional coactivators (Ebert and Bunn 1999, Ebert and Bunn 1998). HNF4α expression in HeLa cells (which do not normally express HNF4α) confers hypoxic inducibility to an EPO reporter gene (Galson et al. 1995, Bunn et al. 1998b, Bunn et al. 1998a).

We examined several important transcriptional regulators of EPO, including HIF1α, HIF2α, ARNT, GATA2 and HNF4α. Prenatal dexamethasone treatment caused a permanent elevation in renal HNF4α (P-1 driven transcripts), whilst expression of other pertinent transcription factors including HIF was unaltered. HNF4α thus

appears to play a causal role in mediating the constitutive increase in renal and fetal hepatic EPO expression observed.

HNF4α may participate in the programming of hypertension yet another mechanism of action. Recently, Dagan et al have shown that the urinary angiotensin II / creatinine level in dexamethasone-programmed offspring is raised in pre-hypertensive rats, even though the plasma angiotensinII level is no different from controls. The urine angiotensinII/creatinine ratio is a measure of the renally produced and secreted angiotensin II, and the authors suggested the intriguing possibility that luminal angiotensin II may be involved in the perpetuation of hypertension in the dexamethasone-programmed rat model of hypertension (Dagan et al 2010). Interestingly, HNF4α is known to be an important transcriptional regulator of renal angiotensinogen (which is the precursor of angiotensin II) and is co-localized with angiotensinogen in the proximal renal tubule. This possibility that HNF4α could be responsible for the raised renal production of angiotensin II observed in this model.

Here we demonstrate, for the first time, that overexposure to glucocorticoids during late gestation causes a lifelong increase in RBC mass in the rat. The other blood cell populations, such as white blood cells and platelets, were unaltered. The elevation in RBC count was associated with increased number of circulating reticulocytes and significant upregulation of mRNA expression and plasma concentration of EPO, the major regulator erythropoiesis, indicating that the increase in RBC mass resulted from enhanced production, rather than prolonged survival of RBCs. The increase in circulating EPO concentration and the resultant change in RBC mass following

prenatal exposure to dexamethasone is therefore likely to be mediated by enhanced EPO production from the kidney (in adults).

There are, however, several limitations of this work. Firstly, it needs to be determined if the increased erythropoeisis observed is through enhanced basal, i.e. bone marrow erythropoeisis or increase in stress erythropoeisis, i.e. extramedullary spleen erythropoeisis. This could be determined by Ter119/CD71 flow cytometric profiles of the bone marrow and spleen. Secondly, although our data show a correlation between HNF4α mRNA levels and EPO expression, this does not prove that HNF4α is causally responsible for increased EPO expression. Further experiments are needed, for instance to localize HNF4α protein within EPO-producing cells, as well as chip analysis from renal extracts with the EPO promoter that unambiguously show increased binding of HNF4α to the EPO promoter/enhancer or an siRNA approach with knock-down of HNF4α in vivo to show that dexamethasone-treated animals no longer show increased EPO expression if their HNF4α expression is attenuated. Finally, dexamethasone programming may also cause possible changes in lung physiology e.g. affecting oxygenation. This would cause HIF proteins to be upregulated causing downstream increased production to be increased. Hence, measures of arterial blood oxygenation and HIF protein abundance should be determined.

In conclusion, the long-term consequences of prenatal glucocorticoid excess on RBC in humans are unknown, but conditions associated with prenatal stress, such as intrauterine growth retardation or maternal diabetes, increase fetal/infant EPO concentrations and erythroblastosis (Lemery et al. 1994, Widness et al. 1981). Our data indicate that RBC mass can be permanently programmed by prenatal stress, and provide a novel mechanism for fetal origins of polycythemia and its associated complications, notably thromboembolic disease.

5.4

SUMMARY

In this chapter, we explored the effect of prenatal dexamethasone on renal EPO and red cell mass. We also observed that parallel changes in HNF4α upregulation. Only HNF4α P-1 driven transcripts were detected in the kidney and dexamethasone-programmed offspring expressed higher levels of the transcripts than their control littermates. This upregulation of HNF4α transcripts may be functionally relevant, at least with regards to renal erythropoietin regulation.

These findings indicate that HNF4α may play an important role in the molecular mechanism of fetal programming of erythropoeisis in our animal model.

CHAPTER 6

6.1

THESIS SUMMARY

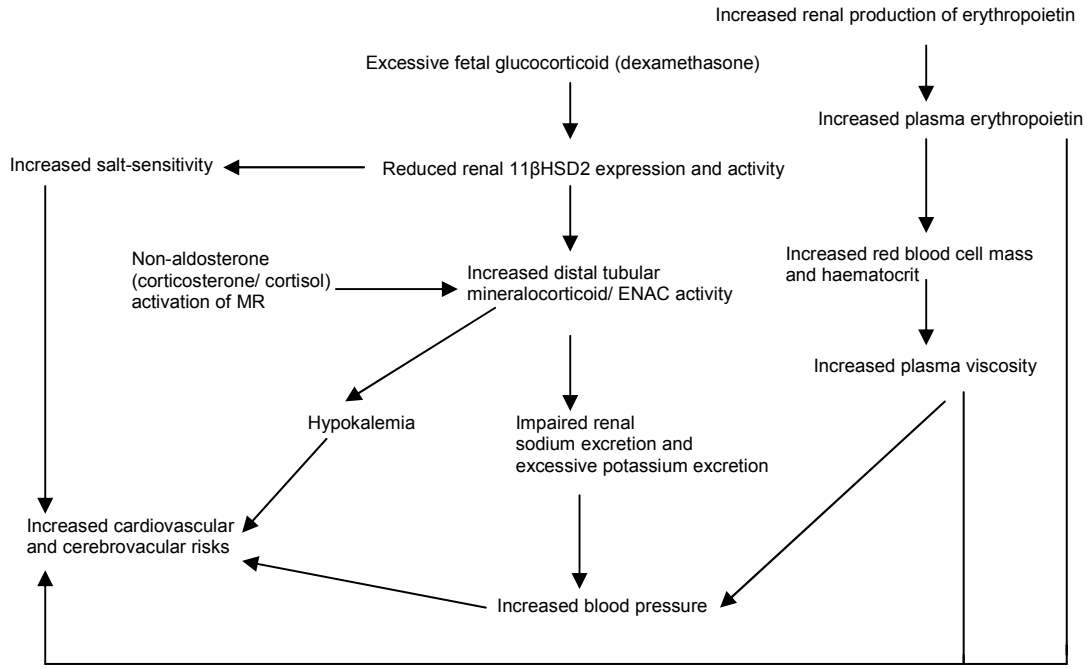

Figure 6-1 Proposed hypothesis and mechanisms by which excessive glucocorticoid (dexamethasone) might cause hypertension, increased salt-sensitivity and ultimately increased cardiovascular risk through key alterations in the kidney- namely, renal distal tubular 11βHSD2 activity and the renal production of erythropoietin.

Fetal programming is thought to subserve an important evolutionary function: to better prepare the fetus for a predicted extrauterine environment. However when there is a gross mismatch between projected ex utero and actual ex utero environment, such as with demographic transition due to increasing urbanization coupled with faster foods and a more sedentary lifestyle, this can result in adverse

outcome, such as deadly epidemics of hypertension, obesity and hyperglycemia (Gluckman, Hanson 2004).

Compelling epidemiological evidence suggests that exposure to an adverse intrauterine environment, manifested by low-birth weight, is associated with cardiometabolic and behavioural disorders in adulthood. Previous work in our laboratory and others have shown in various animal models that this relationship can be mimicked by prenatal treatment with glucocorticoids (Seckl 1997, Benediktsson et al. 1993, Seckl and Meaney 2006, Nyirenda et al. 1998, O'Regan et al. 2004Koenen et al. 2002, Dodic et al. 1998, Levitt et al. 1996, Woods and Weeks 2005). These observations have led to the concept of 'fetal programming' by glucocorticoids. The molecular mechanisms that underlie this relationship remain unclear, but are being extensively investigated using a number of experimental models.

Studies in this thesis have confirmed that fetal over-exposure to glucocorticoids predisposes to hypertension in the affected male adult offspring. We investigated the hypothesis that the kidney plays a central role in the molecular pathogenesis of hypertension in the (dexamethasone) programming model using pregnant Wistar rats and male offspring. Better phenotyping and a better understanding of the pathogenesis of hypertension in programming would be useful in future studies for clinical assessment as well as in rational therapeutics.

We established that renal 11β-HSD2 mRNA expression and activity were indeed decreased (by 45% and 36% respectively) in adult offspring. (Tang et al 2011) The

dexamethasone- programmed offspring also had lower plasma renin concentration, lower 24 hour urinary aldosterone levels and decreased plasma potassium- features consistently seen with the syndrome of apparent mineralocorticoid excess (whereby there is inactivating mutations in the 11β-HSD2 gene) as well as observed in licorice ingestion (due to inhibition of 11β-HSD2 enzyme by licorice). There was also a greater decrease in the urinary ratio of sodium to potassium ratio in response to an exogenous dose of cortisol (when compared to controls) - effects consistent with decreased 11β-HSD2 function in the renal tubules.

Importantly, in a separate cohort of 1 week old neonates, the decrease in 11β-HSD2 mRNA was already found to be present in the dexamethasone-programmed group, implying that the decrease in 11β-HSD2 was likely a primary change due to dexamethasone and not secondary to changes in blood pressure per se. It is particularly important to consider the work of O'Regan et al in this context (O'Regan 2008), wherein it was found that programmed adult rats developed hypotension in the unstressed state (although most other investigators using the more "stressful" tail-cuff methods have found hypertension).

It thus remains a possibility that the downregulation of 11β-HSD2 observed could be a compensatory response to the hypotension observed (it cannot be ruled out that a small but important decrease in blood pressure could be present but undetected at 1 week). Nonetheless, it has been pointed out by the authors (O'Regan et al) that these rats were hypotensive only when left undisturbed in a darkened room. Even minor "stressors" such as switching on the lights, or an observer entering the room caused

the blood pressures of the programmed rats to overshoot those of controls. Hence it is arguable whether under "normal" circumstances in the wild, where escape from predation is stressful and essential for survival; these ever-vigilant rats may indeed have higher blood pressures throughout the day. Additionally, we observed that the renin levels in the programmed rats were lower than controls, and indeed, in most cases of hypertension renin is lowered.

In the offspring that were followed up to be 2 months of age in the younger cohort, it was found that systolic blood pressure in the dexamethasone-programmed group was not different from controls at 2 months of age. However, by stressing these younger animals with high salt diet, the dexamethasone-programmed group not only showed a higher rise in their systolic blood pressures, but they also developed plasma hypokalemia after high salt feeding. Interestingly, it has been observed that salt-sensitivity may be associated with decreased renal 11β-HSD2 activity (Ferrari 2000).

Our data indicate that prenatal dexamethasone programs reduced renal 11β-HSD2 mRNA expression and increased salt sensitivity in the offspring. This increase in blood pressure only became apparent if dietary salt content increases significantly, or with aging, presumably as a result of the additive effects of subsequent environmental factors.

The other novel finding in this thesis was the effect of prenatal dexamethasone on renal EPO and red cell mass. Previous data identified HNF4α, a key transcriptional regulator of EPO, as a key target of glucocorticoid programming in the liver

(Nyirenda 2006). In this thesis, we confirm that HNF4A expression is upregulated in the kidney where it was associated with increased erythropoietin and erythrocyte mass. The degree of red cell mass increase (a mere 6%) is unlikely to cause blood pressure increases. Nonetheless, future studies will be important to uncover the interactions between low birth weight, adaptive response to anemia and the possible contribution of higher erythropoietin levels and erythrocytes to hypertension, hyperviscosity and cardiovascular events (Tang et al, accepted for publication). As with 11β-HSD2, the underlying basis for the change in HNF4α expression is unknown, but epigenetics (e.g. DNA methylation and histone deacetylation) may be involved.

In summary, the work of this thesis agrees with the hypothesis that reduced renal 11β-HSD2 expression provides a novel mechanism for fetal origins of adult salt-sensitive hypertension in our model of programming. The higher erythropoietin levels seen likely does not participate in perpetuation of hypertension via hyperviscosity although it is clinically relevant to cardiovascular mortality. If extrapolated to humans, these data suggest that programmed hypertension may be amenable to measures to reduce features of mild SAME such as blockade of MR or its downstream targets and that such offspring may be specifically sensitive to sodium-induced hypertension.

FUTURE RESEARCH

Despite the advance in our understanding of the dexamethasone model of programming, many unanswered topics still remain. Further work will need to focus on dissecting the exact mechanism of how glucocorticoids programme renal 11β-HSD2. The 11β-HSD2 promoter is known to contain numerous CpG sites. Methylation of these CpG sites are known to correlate with gene expression: the more the methylation, the less the gene expression (Alikhani-Koopaei et al. 2004). Numerous new techniques, including high through-put bisulfite sequencing (Laird 2005, Gitan et al. 2002, Cokus et al. 2008) are rapidly evolving currently, presenting an opportune time to study if methylation patterns in the in utero environment change with glucocorticoid exposure, and if glucocorticoid exposure in early life influences the rate of methylation with aging. Intriguingly, there is already early evidence that methylation of the11β-HSD2 promoter can be programmed by maternal uterine artery ligation (Baserga 2010).

Additionally, an extension of our work done will be to investigate if 11β-HSD2 in other important cardiovascular structures relevant to blood pressure control, such as the vascular and cardiac, is similarly down-regulated. Recent work has shown that 11β-HSD2 may play an important role in limiting non-aldosterone activation of MR (by corticosterone) (Alzamora, Michea and Marusic 2000, Brown et al. 1996). Excessive MR activation may lead to vessel fibrosis, accelerated artherosclerosis and increased vascular oxidative stress, exacerbating endothelial dysfunction, hypertension, vascular and renal disease (Young, 2003).

It will also be important to investigate if the rise in systolic blood pressure is of pathophysiological significance in our model of programming. Here, using new techniques such as the optical coherence tomography (Brezinski 1996), direct assessment of vascular atherosclerosis (including the coronary vessels), for instance after high fat feeding, will be of value in future animal work.

Finally it would be important to investigate if the effect of glucocorticoid programming on red blood cell mass is similarly observed in human populations. Animal studies in quantifying the erythropoietic and bone marrow response to anemia and hypoxia will also shed light on whether the findings of this thesis is of physiological significance with regards to the adaptative response to anemia, an endemic condition especially in the developing world.

6.2

IMPLICATIONS OF WORK

6.2.1

POSSIBLE IMPORTANCE OF 11 BETA-HYDROXYSTEROID DEHYDROGENASE TYPE 2 IN HUMAN HYPERTENSION

Our experiments have shown that glucocorticoids in the form of dexamethasone programmed permanent decreases in 11β-HSD2 mRNA and activity in the adult offspring. The degree of reduction in 11β-HSD2 enzyme observed was associated with low-renin (low-aldosterone) type systolic hypertension. Moreover, younger dexamethasone-programmed offspring displayed salt-sensitive hypertension.

25% of all human hypertension is of the low-renin type (Buhler et al. 1984). What is less clear is the proportion of low-aldosterone low-renin type hypertension in the population. This proportion of low-renin hypertension parallels the proportion of low birth weight infants distributed in the normal Gaussian manner. Future developments in developing accurate measures of renal 11β-HSD2 activity will help resolve the question if 11β-HSD2 play a significant role in human hypertension.

If these physiological changes were similar in low birth weight humans (Simonetti 2008), it would have important implications. Firstly, that children born in

impoverished societies (a substantial proportion of the world's population) are at risk of developing (salt-sensitive) hypertension. Ironically, it is the children under poorer socioeconomic circumstances that are consuming the greatest amount of salt on a daily basis (Gray 2009). Moreover, as economies develop in third world countries, these children, already prone to developing hypertension, will be subjected to the industrialized lifestyles which will compound the possible epidemic in incidence of hypertension in these societies. Currently, the hypertension epidemics in India and China, the 2 most populous countries in the world, are possible consequences of thrusting industrialized lifestyles upon hypertension-prone formerly low birth weight babies in these societies.

The current trend of excessive salt intake in almost all but the most primitive of societies will assume a greater importance in public health policy if the findings of our work are extrapolatable to humans. It will be important to test the hypothesis that decreasing the average consumption of salt will lead to a decrease in the incidence of hypertension and its consequences, although this will require further rigorous scientific evidence, trials and political (and personal) will.

6.2.2

IMPLICATIONS FOR PERSONALIZED MEDICINE AND THERAPEUTICS

One of the aims of medical genetics is accurately predicting of the risk of developing disease. Will it ever be possible to predict disease e.g. hypertension?

As argued by Weatherall using beta-thalassemia as an example, even if a disease were purely genetic, there are still primary, secondary and tertiary genetic modifiers of phenotype (Weatherall 2001). The resultant permutations and combinations of the various modifying genes inherited make the concept of predicting phenotype using genotype look overly simplistic, at the very least.

Our research shows that the in-utero environment is able to alter gene expression (11β-HSD2) and influence resultant offspring phenotype. This adds another layer of complexity in trying to predict phenotype- the epigenome. Indeed, for the case of hypertension, genes are thought to account only 20-60% the risk of developing hypertension; the environment playing a (probably) greater part than previously thought (Lifton 2001).

Until we know exactly how the environment affects the expression of genes (e.g. through epigenome), we are far from accurately predicting disease risk. To make matters even more complex, epigenome has been observed to change over time. For instance, the epigenome of twins were found to diverge over the years, indicating the

possibility that the environment may play a crucial role in the rate of CpG methylation and other crucial epigenomic processes (Fraga 2005). All these suggest the near-impossibility of predictive testing given the myriad complexities of genotype-environment-genotype interactions.

On the other hand, if research can clearly define the many factors in accounting for disease development, then it may just be possible in future to compute and accurately predict risk for each individual. Future research, especially in the field of epigenetics, should help inform and enlighten us.

6.2.3

IMPLICATIONS FOR THERAPEUTICS OF HYPERTENSION

The 7th Joint National Council (JNC) guideline on hypertension has singled out thiazide diuretics as a first line drug for hypertension treatment, other drugs such as ACE-inhibitors, beta-blockers, calcium channel blockers usually started if there are compelling indications (Chobanian et al. 2003).

Our work in dexamethasone-programmed rats suggests that hypertension in these animals is due to increased mineralocorticoid receptor activation (by corticosterone in the face of decreased renal 11β-HSD2 activity) leading to low-renin type hypertension with hypokalemia. If this mechanistic finding is extrapolatable to humans, it is plausible that low birth weight (often recallable by patients) may be a compelling indication for use of mineralocorticoid receptor antagonists (such as spironolactone, eplerenone and amiloride) at an earlier stage of hypertension, leading to more rational therapeutics.

In addition, hypokalemia provoked by the mineralocorticoid antagonists is an added advantage: potassium is known to be an independent risk of cardiovascular and stroke, two of the more feared complications of hypertension (Khaw and Barrettconnor 1987, Meneely and Battarbee 1976, Nordrehaug, Johannessen and Vonderlippe 1985, Savage et al. 1998)

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6.2.4

IMPLICATIONS OF THE PROGRAMMING OF RED BLOOD CELL MASS

We hypothesized that glucocorticoid programming might have salient effects on other physiological systems (e.g. hematological system). These coordinated changes in whole organism physiology would allow the offspring to better survive an adverse environment ex-utero.

Our work showed that red cell mass, hemoglobin and hematocrit is increased in dexamethasone-programmed offspring, a novel finding. This work has important implications in providing mechanistic insights into how individuals have marked variable response to anemia.

Millions of low birth weight infants in the third world suffer from anemia caused by malaria or hereditary diseases such as the thalassemias and sickle cell disease. Further study into the molecular basis on how programming might allow them to adapt and augment their response to anemia (e.g. via erythropoietin and HNF4α) could provide new pathways for rational treatment.

On the other hand, raised hematocrit has been implicated in strokes and acute cardiovascular events. Hence, exploring the pathways regulating the hematopoietic response may yield fruitful therapeutic targets in reducing cardiovascular and stroke morbidity and mortality in the future.

6.3

CONCLUSION

GLUCOCORTICOID PROGRAMMING: PROPOSING AN EXTENDED MODEL INVOLVING 11 BETA-HYDROXYSTEROID DEHYDROGENASES

Dexamethasone programming in pregnant Wistar rats produces offspring with features of the metabolic syndrome, namely hypertension and hyperglycemia (Nyirenda 1998. O'Regan 2004).

Glucocorticoids are important key metabolic regulators, at least in mammalian physiology. Previously, the role of the glucocorticoid receptor as a key programming target in various tissues has been comprehensively reviewed (Seckl 2004). What is emerging, unsurprisingly, is that the 11β-HSDs are also important programming target genes (see Figure 6-1).

As mentioned in the earlier chapters, the enzymes 11β-HSD1 and 11β-HSD2 mediate intracellular glucocorticoid action. 11β-HSD1 amplifies intracellular glucocorticoid action by favoring the conversion of inactive cortisone to active cortisol (in humans). Transgenic mice model overexpressing 11β-HSD1 adipocyte cells, developed insulin-resistant glucose intolerance and other features of Cushing's syndrome, even though plasma cortisol were not different from control animals (Masuzaki 2001). Recently, we have shown that in marmoset primates, dexamethasone given in late

gestation was associated with increased liver expression of 11β-HSD1 (Nyirenda 2009). This local amplification of glucocorticoid action was associated with increased expression of PCK1, a rate-limiting gluconeogenic enzyme upregulated by the increased local cortisol levels, leading to the glucose intolerance observed in the dexamethasone-programmed marmoset offspring.

In this thesis, we demonstrate the importance of renal 11β-HSD2 in the dexamethasone programming model in rats. More work will surely be needed to see if the decrease in 11β-HSD2 activity in the kidney is present in other species e.g. marmosets and most importantly, in low birth weight humans. Also, it has yet been determined if renal 11β-HSD2 is important in the pathogenesis of other models (e.g. maternal low-protein diet, uterine artery ligation), although there is emerging evidence that this may be a common programming target gene in the pathogenesis of hypertension in these other models of prenatal insults (Bertram C, 2001; Baserga M, 2010).

The finding that decreased renal 11β-HSD2 activity may be important in the pathogenesis of hypertension is well supported by physiology. Arthur Guyton and Richard Lifton have both argued convincingly from their work that for blood pressure to be elevated, the kidneys must somehow be involved by increasing the net sodium and water reabsorption (Guyton, 1991; Lifton 2001). With decreased renal 11β-HSD2, which was shown to reside mainly in the distal tubules, corticosterone (the active glucocorticoid in rats), because of its high affinity for the mineralocorticoid receptor, might theoretically transactivate the mineralocorticoid

receptor leading to the molecular cascade ending in a higher net reabsorption of sodium and water, leading to hypertension as mentioned in the earlier chapters.

It seems logical that the 11β-HSDs are key targets in programming (see Figure 1). By controlling tissue-specific glucocorticoid activation or inactivation, they allow local regulation of glucocorticoid action largely independent of the level of circulating cortisol, thus enabling the different tissues to fine-tune and adjust cortisol action independently through their varying levels of the 11β-HSDs. In our experiments, we found that only 11β-HSD2 mRNA expression was altered in the kidneys, and not in the other tissues examined – liver and adrenals. The mechanism of tissue-specific programming was not addressed in this thesis but it may involve perturbations of tissue specific transcriptional factors operative during critical window periods of organ development.

In conclusion, it appears that glucocorticoid programming results in many coordinated metabolic changes in physiology. Many of these changes have already been shown to be mediated by changes glucocorticoid action- through altering levels of the glucocorticoid receptor, 11β-HSD1 and 11β-HSD2. We propose that these are important targets to explore in the other programming models and potentially relevant to humans.

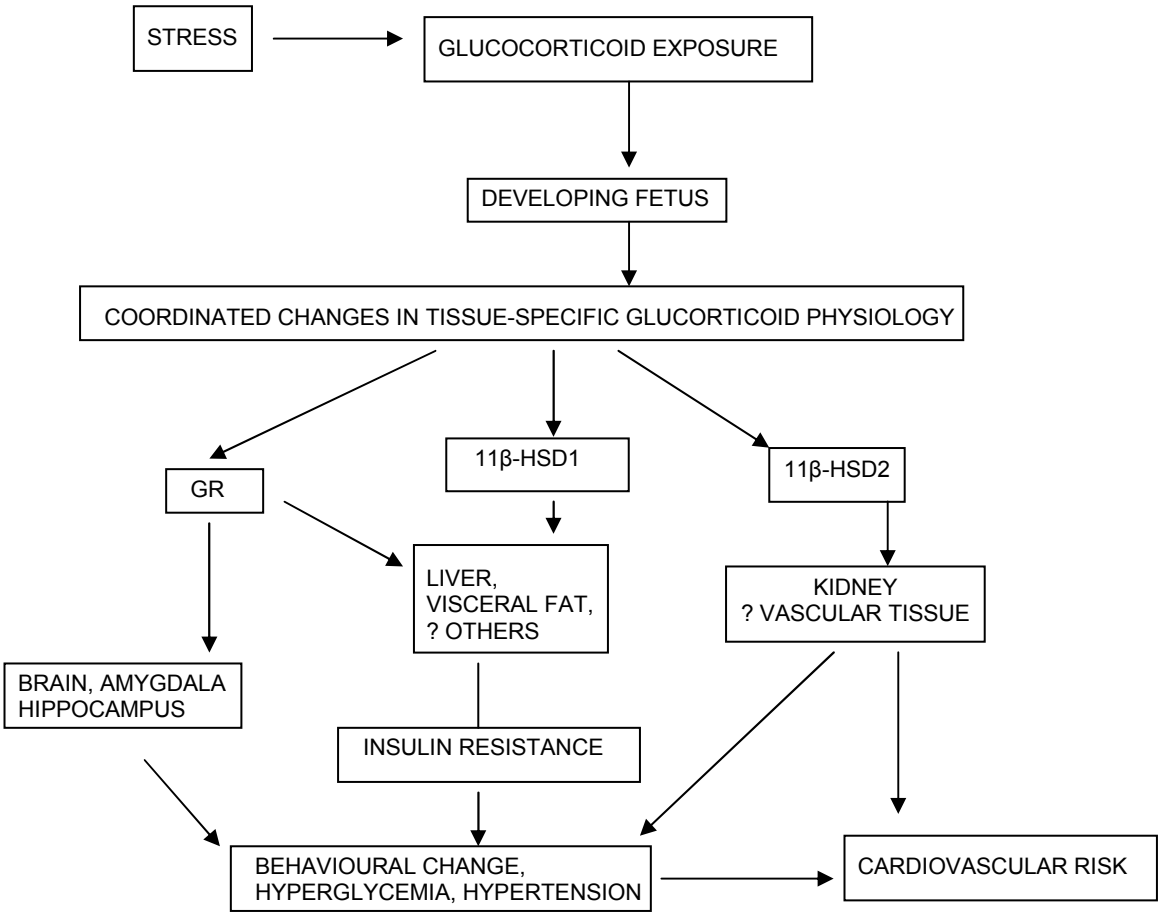

Fig 6-2 Metabolic Changes resulting from glucocorticoid programming

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